HYDROSULFITES #60

Hydrosulfates #60 10/12/73

GRAS MONOGRAPH SERIES

HYDROSULFITES

prepared for
THE FOOD AND DRUG ADMINISTRATION
DEPARTMENT OF HEALTH, EDUCATION
AND WELFARE

OCTOBER 12, 1973

This publication was prepared under Contract Number FDA 72-100
with the Public Health Service, Food and Drug Administration,
Department of Health, Education, and Welfare

prepared by
Tracor Jitco, Inc.

CHEMICAL INFORMATION

BIOLOGICAL DATA

BIOCHEMICAL SECTION

Foreword

Due to the small number (32) and non-pertinency of the reference cards supplied, Tracor Jitco performed a manual search of the following sources to obtain additional data regarding the safety of sodium and zinc hydrosulfite:

Chemical Abstracts, 1920-January 1973
FDA petitions
Food Codex
Comprehensive GRAS survey, NAS/NRC 1972
Toxicity Bibliography, 1968-current
Reports of the "Joint FAO/WHO Expert Committee on Food Additives"
Toxic Substances List, 1973 Edition (preliminary)
Survey of Compounds Tested for Carcinogenic Activity;
National Cancer Institute, U.S. Dept. of Health, Education, and Welfare.
Agricultural Statistics, 1972
Handbook of Food Additives, Chemical Rubber Co.

The search terms used were:

Sodium hydrosulfite
Zinc hydrosulfite
Sodium dithionite
Zinc dithionite
Dithionous acid, disodium salt
Dithionous acid, zinc salt
Sugar manufacture

In addition, the following information gathering steps were taken:

a. A computer search was also conducted at our request by the Toxicology Information Response Center at Oak Ridge, Tennessee. This search covered:

Chemical Abstracts, Vol. 1-75 (1907-1971)
Biological Abstracts, Vol. 1-53 (1927-1972)
Advances in Food Research, Vol. 1-17 (1948-1969)
Excerpta Medica XXX (Pharm. & Tox.) Vol. 18-25 (1965-1972)
Nutrition Abstracts and Reviews, Vol. 1-41, (1931-1971)
Science Citation Index (1970-1971)

Medline 1969-1973 (Apr.)
Toxicon
CBAC Data Base, Vol. 15-17(5) (1972-1973)
Biological Abstracts Data Base (1971-1972)
BioResearch Index Data Base, Vol. 71-73(1) (1971-1973)

b. The Sugar Information Center in New York City was contacted but no information was available after a search.

c. Some information indicated that Upjohn had conducted some experiments on the toxicity of sodium hydrosulfite. We requested information from Upjohn but did not receive a reply. No other work had been done on the subject.

d. The Manufacturing Chemists Association was also asked for information, but none was obtained.

Only a small number of additional pertinent references were found; these are included in the bibliography section of this monograph.

CHEMICAL INFORMATION

SODIUM HYDROSULFITE

I. Nomenclature

A. Common names: none

B. Chemical names: Sodium hydrosulfite, Sodium dithionite, Sodium sulfoxylate

C. Trade names: Vatrolite, Lykopon

D. Chemical Abstracts Services Unique Registry Number: 7775146

II. Empirical Formula

$Na_2O_4S_2$

III. Structural Formula

$Na_2S_2O_4$

IV. Molecular Weight: 174.13

V. Specifications

Not available.

VI. Description

A. General characteristics

Sodium hydrosulfite is a free-flowing white to grayish white crystalline powder with a slight characteristic odor.

B. Physical properties

Sodium hydrosulfite is extremely soluble in water and slightly soluble in alcohol.

C. Stability

Sodium hydrosulfite oxidizes in air (more so when moisture is present) to bisulfite and bisulfate. It is stable in alkali but decomposes in acid.

VII. Analytical Methods

A rapid, semi-quantitative method for detection of hydrosulfite ion is presented by Sabo (44) in which a solution containing the anion is spotted on a silver chromate impregnated paper. A change in color from red to black indicates $S_2O_4^{2-}$ and the test is sensitive to 1.0 μg in a 0.04 ml drop. The sulfide anion (S^{2-}) also gives a black spot, and of those metals which form insoluble chromates, only mercury and lead are reported to interfere.

ZINC HYDROSULFITE

I. Nomenclature

A. Common names: none

B. Chemical names: Zinc hydrosulfite, Zinc dithionite

C. Trade names: Protolin Z

C. Chemical Abstracts Services Unique Registry Number: 7779864

II. Empirical Formula

O_4S_2Zn

III. Structural Formula

ZnS_2O_4

IV. Molecular Weight: 193.50

V. Specifications

VI. Description

A. General characteristics

Zinc hydrosulfite is a free-flowing white non-dusting powder with a slight SO_2 odor. (43)

B. Physical properties

Zinc hydrosulfite is extremely soluble in water.

VII. Analytical Methods

See information presented for sodium hydrosulfite.

BIOLOGICAL DATA

I. Acute Toxicity

A. Rats

Heyl and Greer (27) conducted a series of acute experiments on white rats (strain, age, sex, and number not given). The results are tabulated in Table 1. In these tests Heyl and Greer observed that sodium hydrosulfite (83%) dissolved in water and injected intravenously into white rats at dosages of 25-50 mg/kg BW produced no effects. At 100 mg/kg the rats showed signs of labored breathing within a few seconds after the beginning of the injection. This effect subsided within 5-10 minutes and the rats appeared normal thereafter. At 150 mg/kg the rats showed signs of suffocation. At 210 mg/kg, dyspnea became acute and the rats "doubled up" with convulsion within 3-5 minutes. This effect subsided within 8-10 minutes and there were no delayed symptoms.

B. Dogs

Bond and Gray (07) administered orally as much as 1 g/kg BW sodium hydrosulfite to dogs (age, sex, and number not given) without any apparent injurious effect. No details of the experiment were given.

II. Short-Term Studies

No information on the short-term toxicity of sodium or zinc hydrosulfite has been found in the literature.

III. Long-Term Studies

No information on the long-term toxicity of sodium or zinc hydrosulfite has been found in the literature.

IV. Special Studies

Mutagenic Tests

Prozorov (40) studied the effect of sodium hydrosulfite on the mutation rate of the unstable strain of Bacterium Prodigiosum. A stock strain of Bacterium Prodigiosum was grown on agar with 1% glucose. Sodium hydrosulfite (0.05%) was added to the agar and the mutation rate per generation was counted. Sodium hydrosulfite was found to decrease natural mutagenesis (see Table 2).

Table 1. ACUTE TOXICITY (27)

(Injected, white rats, sex and number not specified)

SUBSTANCE and PURITY*	DOSAGE mg/kg Body wt.	OBSERVATION
Sodium hydrosulfite	150	Not fatal
(83%)	180	Not fatal
	210	Not fatal
	240	Fatal in 5 min.
(83.5%)	100	Not fatal
	125	Not fatal
	150	Fatal in 7 min.
(28%)	50	Not fatal
	100	Not fatal
	150	Not fatal
	200	Fatal in 4-10 min.
(14%)	200	Not fatal
	300	Not fatal
	325	Not fatal
	350	Fatal in 4 min.
Unspecified Purity	25-50	Not fatal
	100	Not fatal
	150	Not fatal

* Purity is measured by % of theoretical reducing power; (see original paper for details of determination)

Table 2. Mutagenic Tests (40)

Size of Sector	Rate of mutation per generation	
	Control	Sodium hydrosulfite
1/2	10,0 ± 1,6	0,8 ± 0,35
	σ = 3,2	σ = 1,1
	n = 20	n = 36
1/4	7,6 ± 0,8	0,7 ± 0,23
	σ = 1,6	σ = 0,7
	n = 20	n = 36
1/8	6,7 ± 0,8	0,7 ± 0,13
	σ = 1,7	σ = 0,45
	n = 20	n = 36

BIOCHEMICAL SECTION

I. Breakdown

Sodium hydrosulfite will oxidize to sodium bisulfate and sodium bisulfite in the presence of air, and moreso when moisture is present (49 ,43). In dilute solutions, hydrosulfites tend to break down with the formation of SO_2 and colloidal sulfur (50).

Irwin Stone (50) has examined the use of sodium hydrosulfite for the removal of dissolved oxygen in beer in combination with sodium isoascorbate. The hydrosulfite-isoascorbate complex formed contained 25% sodium hydrosulfite and 75% sodium isoascorbate. The isoascorbate stabilized the hydrosulfite ion so that the rapid production of SO_2 and colloidal sulfur is not observed while the solution retains its antioxidant properties.

Under the conditions of pH, temperature, concentration and other factors existing in the treatment of beer, the reaction of hydrosulfite with molecular oxygen proceeds according to the equation:

$$Na_2S_2O_4 + 1/2\ O_2 + H_2O \rightarrow 2\ NaHSO_3$$

as given by Stone.

Expecting the production of sodium bisulfite to lead to an increase in the amount of SO_2 in beer, Stone (50) tested for such an increase. Beer to which 40 ppm of the hydrosulfite-isoascorbate complex was added showed a free SO_2 concentration of 0.8 ppm, an increase of 0.5 ppm over 0.3 ppm free SO_2 found in untreated beer. Table 3 shows the effect of storage on SO_2 content of beers treated with the hydrosulfite-isoascorbate complex.

Table 3 (50)

EFFECT OF STORAGE ON SULFUR DIOXIDE CONTENT OF BEERS

Added Na Dithionite-Na Isoascorbate ppm	Air Content of Bottles ml/12 oz	"Free" Sulfur Dioxide Initial ppm	1 month ppm	2½ months ppm
None	1	1.3	1.0	0.7
40	1	4.6	3.0	1.6
None	6	1.2	nil	nil
40	6	5.1	1.0	0.2

Stone (50) also tested for the rate of disappearance of any unreacted hydrosulfite remaining in the beer. In general, he found that at above the freezing temperature of beer, the hydrosulfite is unstable and rapidly changes to sulfite. Table 4 shows the effect of temperature and storage time on residual hydrosulfite. Figure 1 graphically displays the disappearance of hydrosulfite from beer at varying temperature.

Table 4 (50)

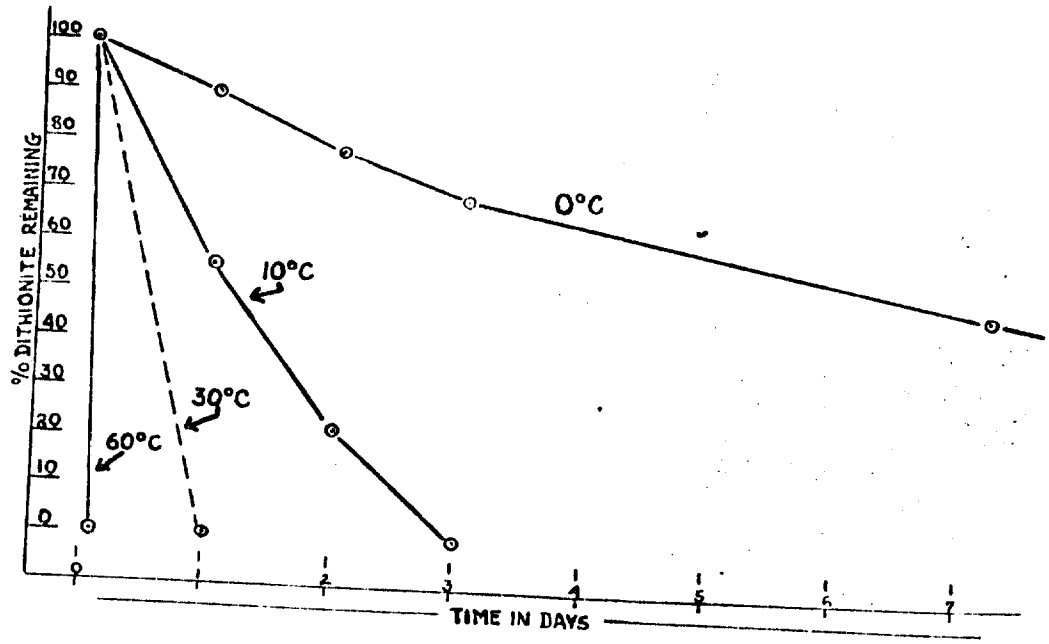
THE DITHIONITE ANION IN BEER OF DIFFERENT TEMPERATURES

Beer	Added* Sodium Dithionite ppm	Storage Temperature °C	Residual Sodium Dithionite Found: Immediately after Addition ppm	After 1 day ppm	After 2 days ppm	After 3 days ppm	After 1 week ppm
Unpasteurized Prefiltered Beer							
A	29	0	9	8.5	7.5	6.5	4.0
A	29	10	9	5.0	1.5	0.0	0.0
A	29	30	9	0.0	0.0	—	—
B	29	0	11	9.0	8.0	6.5	5.0
B	29	10	11	5.0	1.5	0.0	0.0
B	29	30	11	0.0	0.0	—	—
C	39	0	14	13.0	11.0	10.5	7.5
C	39	10	14	9.0	5.0	0.0	0.0
C	39	30	14	0.0	0.0	—	—
Pasteurized			Immediately after Pasteurization				
A	29		0.0	0.0			
B	29		0.0	0.0			
C	39		0.0	0.0			

*Added as a mixture of Na Dithionite-Na Isoascorbate (Isona) 25 + 75.

Figure 1 (50)

Average time of disappearance of dithionite anions from beer.



II. Absorption and Distribution

There is no information in current literature concerning the absorption and distribution of sodium or zinc hydrosulfite in animals or humans.

III. Metabolism and Excretion

There is no information in current literature concerning the metabolism and excretion of sodium or zinc hydrofulfite in animals or humans.

IV. Effects on Enzymes and Other Biochemical Parameters

A. In Vitro

1. Basu and Chakravarty (04) studied the inhibiting effect on sodium hydrosulfite on the enzymes trypsin and papain.

Trypsin, in the presence of cysteine as activator, was subjected to 1/4000 concentration of sodium hydrosulfite at 37°C, pH = 8.67. After one hour, peptone was added to the solution, reducing the sodium hydrosulfite concentration to 1/10,000. No inhibition of peptone hydrolysis was observed after 35 minutes, while 7% inhibition occurred after 65 minutes, showing that the absorption of sodium hydrosulfite on the trypsin surface was very slow and unappreciable for the first 2 hours.

Similarly, papain, also in the presence of cysteine as activator, was subjected to a 1/4000 concentration of sodium hydrosulfite at 37°C. The solution was at pH = 5. After one hour, gelatin was added reducing the sodium hydrosulfite concentration to 1/10,000. No inhibition of gelatin hydrolysis by papain was observed after 2 hours.

2. Meerson and Shleifer (38) investigated the effect of sodium hydrosulfite on the hydrolytic activity of the pancreatic and hepatic lipases. They found that sodium hydrosulfite did not inhibit the hydrolytic activity of lipase. No details of the experiment were given.

3. Myers and Slater (39) found that in aged and frozen mitochondria, sodium hydrosulfite produced marked activation of ATPase between pH 6 and 9. Maximum stimulation was observed at pH 7.5 with 10^{-3} M $Na_2S_2O_4$. At pH above 9, ATPase activation by sodium hydrosulfite was negligible, even when the concentration of the hydrosulfite was increased to 10^{-2}M.

There was no activation of ATPase activity in normal mitochondria in the presence of 10^{-4}M DNP. However, the authors note that the experiment was not conclusive since the hydrosulfite changed the color of DNP from yellow to orange.

The effect on ATPase by sodium hydrosulfite was not observed in other reducing agents, including glutathione and ascorbic acid.

4. Caldwell and Seegers (11) tested the effect of sodium hydrosulfite on the activity of thrombin, prothrombin and autoprothrombin C. One part of the hydrosulfite was mixed with one part of the protein so that the final concentration of the hydrosulfite was 0.05 M. An aliquot was taken immediately and the activity determined. The value found was taken as 100% activity. The mixture was then kept at room temperature and the activity tested again after 2 and 24 hours. The results are shown in Table 5.

Table 5

Effect on the Activity of Prothrombin, And Autoprothrombin C by Sodium Hydrosulfite (11)

	Prothrombin		Thrombin		Autoprothrombin C	
	2 hrs	24 hrs	2 hrs	24 hrs	2 hrs	2 hrs
% Activity Remaining	24%	24%	90%	25-50%	24%	24%

5. Several workers studying the kinetics of hemoglobin deoxygenation have used sodium hydrosulfite (33 , 12 , 24 , 5). Legge and Roughton (33) found that at pH 6.8 the dissociation of oxyhemoglobin in the presence of 0.1% sodium hydrosulfite occurred in two

phases. A rapid dissociation from 100% O_2Hb to 20 ± 10% O_2Hb, considered a direct effect of oxygen uptake by the hydrosulfite, and a slower phase which the authors considered to be an effect of the hydrosulfite oxidation products acting on the hemoglobin.

Sirs (47) extended the work to the study of the rate of egress of oxygen from sheep erythrocytes. Placing the whole erythrocytes in a solution containing 0.075-0.35mM sodium hydrosulfite, at 10° the rate of diffusion of oxygen across the membrane resulting from hemoglobin deoxygenation was observed. Sirs quoted others (33) as having shown that the membrane is impermeable to the hydrosulfite ion thus ruling out direct interaction of the hydrosulfite with O_2Hb. He suggested that the hydrosulfite kept the effective level of O_2 outside the cell membrane at zero, resulting in dissociation of oxyhemoglobin in the erythrocyte and diffusion of the oxygen across the cell membrane.

B. In Vivo

Raunio and Lilius studied the effects of sodium hydrosulfite on enzymes in vivo using Escherichia coli (41). When sodium hydrosulfite was added to the growth medium at a concentration of 5 mM at the beginning of the logarithmic phase of growth (when the specific activity of isocitrate dehydrogenase is highest), the authors found that there was no decrease in isocitrate dehydrogenase activity, even 18 hours after addition.

When sodium hydrosulfite (5 mM) was added to the medium at the beginning of the logarithmic phase, the specific activity of leucine aminotransferase did not rise as high as in untreated controls. Under normal conditions, the specific activity of this enzyme is highest at the end of the logarithmic phase, and decreases in the stationary phase. Later addition led to a sharp increase in activity followed by a decrease, though not as marked as in normal growth.

In testing the effect on NADH oxidase, sodium hydrosulfite (5 mM) was added to different cultures at different points on the growth curve. It was added to one culture at the beginning of the

logarithmic phase, to another during the logarithmic phase, and to a third at the end of the logarithmic phase. The specific activity of the enzyme was found to be lower in all three instances, though the difference from the normal was less when the addition of the hydrosulfite was later.

The specific activity of alanine aminopeptidase did not decrease at the end of growth in the normal culture. With sodium hydrosulfite added at 5 mM, the specific activity of the enzyme decreased for a short time, and then increased. When the hydrosulfite was added at the beginning of the stationary phase, there was no significant difference in activity from the normal.

The authors concluded that when sodium hydrosulfite was added to the culture medium before the synthesis of a particular enzyme was proceding at its maximal rate, the activity of the enzyme remains lower owing to inhibition of its synthesis by the hydrosulfite.

V. Drug Interaction

A. Bacteria

Bondi, et al. (08) showed that sodium hydrosulfite reduced the bacteriostatic activity of streptomycin on Escherichia coli. At a final concentration of 0.05% sodium hydrosulfite in tryptone broth, 16 units/ml of streptomycin was necessary for inhibition, compared to 2 units/ml in the tryptone control.

B. Dogs

Bond and Gray (07) experimented with sodium hydrosulfite in acute arsenic poisoning. This was based on previous findings by co-workers that sodium hydrosulfite, if given immediately following the oral administration of a fatal dose of mercuric chloride, would save the animal.

Healthy dogs, after a 24-hour period of starvation, were fed 1 ml/kg BW (twice the minimal fatal dose) of potassium arsenite solution orally. This was followed by the administration of 100 mg/kg of sodium hydrosulfite in the form of a 10% solution, which

was followed immediately by 25 ml of N hydrochloric acid. Only those dogs given sodium hydrosulfite immediately (0-5 minutes) after the poisoning survived (see Table 6).

Dogs fed 0.75 ml/kg of arsenic solution and treated with 100 mg/kg sodium hydrosulfite within 10 minutes of the poisoning survived (see Table 7).

Table 6

Results Following the Immediate Use of Sodium Hydrosulfite in Poisoning from the Oral Administration of Potassium Arsenite Solution (07)

Weight of dog, kg	Solution of Potassium Arsenite-U.S.P., ml/kg	Sodium Hydrosulfite mg/kg	Result
10.1	1	100	Survived *
7.27	1	0	Died, 8 hours
5.0	1	100	Survived *
6.6	1	0	Died, 9 hours

* If animal was living at the end of two weeks, it was reported as survived.

The authors believed that if the dogs had food in their stomach, absorption of arsenic would be delayed to an extent that the period of sodium hydrosulfite's effectiveness would be prolonged. Although the authors did not recommend the use of sodium hydrosulfite in place of gastric lavage for the treatment of acute oral arsenic poisoning, they did suggest that immediate administration of appropriate amounts of sodium hydrosulfite following arsenic poisoning may render the arsenic unabsorbable until thorough gastric lavage can be effected.

Table 7. Results Following the Use of Sodium Hydrosulfite in the Treatment of Dogs Poisoned by the Oral Administration of Potassium Arsenite Solution (07)

Weight kg	Arsenic Solution ml/kg	Sodium Hydrosulfite, mg/kg	Time Interval in minutes	Result 24 hours
12.2	1	none	---	Dead
12.4	1	none	---	Dead
10.8	1	none	---	Dead
4.37	1	none	---	Dead
14.0	1	100	30	Dead
13.5	1	100	30	Dead
5.0	1	100	30	Dead
3.45	1	100	30	Dead
4.8	1	100	4.75	6 days
5.1	1	100	4.25	Survived
15.8	1	100	3	Survived
3.75	1	100	3	Survived
10.3	1	100	10	Dead
11.7	1	100	10	Dead
13.7	1	100	10	Dead
11.4	1	100	9.25	Dead
4.0	0.75	none	---	Died, 48 hours
3.86	0.75	none	---	Died, 8 hours
3.8	0.75	none	---	Died, 24 hours
6.05	0.75	none	---	Died, 24 hours
8.9	0.75	none	---	Died, 24 hours
10.9	0.75	none	---	Died, 24 hours
14.6	0.75	none	---	Died, 24 hours
18.7	0.75	none	---	Died, 24 hours
5.7	0.75	100	5	Died, 36 hours
6.1	0.75	100	5.75	Survived
13.05	0.75	100	6	Survived
10.3	0.75	100	6	Survived
8.07	0.75	100	6	Survived
8.7	0.75	100	6	Survived
8.5	0.75	100	10	Survived
8.4	0.75	100	10	Survived
6.9	0.75	100	10	Survived
8.3	0.75	100	10	Survived
8.68	0.75	100	10	Survived
7.7	0.75	100	15	Died, 24 hours
7.9	0.75	100	15	Survived
8.5	0.75	100	16	Died, 24 hours
5.3	0.75	100	16	Died, 24 hours
3.66	0.75	100	20	Died, 48 hours
4.1	0.75	100	20	Died, 24 hours
6.7	0.75	100	11	Survived

VI. Consumer Exposure

Sodium hydrosulfite has been shown to be effective in the prevention of melanosis in frozen and ice-stored crustaceans. In the study on board trawlers, (15), it was found that crustaceans kept under ice were free of melanosis after 9-18 days when immersed in a 7-15% solution for 3-15 minutes. Where crustaceans were frozen, immersion for 2-5 minutes in 3-7% solutions before freezing resulted in no melanosis after 5-13 weeks.

Stone (50) has shown that a solution containing 25% sodium hydrosulfite and 75% sodium isoascorbate is effective as an anti-oxidant in beer. When the mixture was used at a level of 40 ppm, the hydrosulfite effectively removed all dissolved oxygen and had a strong effect in lightening the beer. No residual hydrosulfite ion was found in the finished beer because of its instability above freezing temperatures.

No data was available on the extent of usage of sodium hydro-sulfite in either of the above food processes.

The literature searched contained no reference to zinc hydro-sulfite in food processing.

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Action of Dyestuffs and Narcotics on Proteolytic Enzymes. Trypsin and Papain.

By Kalipada Basu and Ramakanta Chakravarty.

The purification of many enzymes has reached a very high stage and in the case of some enzymes like urease (Sumner, *J. Biol. Chem.*, 1926, 69, 435) and pepsin (Northrop, *J. Gen. Physiol.*, 1930, 13, 739, 767), it is claimed that they have been obtained in the pure crystalline form. But very little is known about the constitution of the enzymes. An insight into the constitution and mode of action of the enzymes could be obtained if the nature of the active group or groups in the enzyme could be determined. Toxic actions of substances of known structure on an enzyme would give us some information about the nature of the active group in the latter. Investigations in this line have been begun by Bamann and Schmelter (*Z. physiol. Chem.*, 1931, 197, 1) who showed that indicators of the type phenolphthalein, bromothymol blue, etc., have a toxic action on lipase. Quastel (*Biochem. J.*, 1931, 25, 628, 898; 1932, 26, 1665) has tried the action of a series of acidic and basic dyes on dehydrogenase, fumarase and urease, and finds that generally the inhibiting action of the dyestuffs is limited to the basic dyestuffs. It should thus be possible to distinguish between apparently closely related enzymes like the different proteinases, polypeptidases, etc., by taking advantage of the specific toxic action of the dyestuffs.

The first observation on the action of dyestuffs on proteolytic enzymes was made by Marston (*Biochem. J.*, 1923, 17, 851) who found that dyes of the safranine type precipitated the proteolytic enzymes, the precipitates being proteolytically active.

The purpose of the present investigation is to attempt to elucidate the nature of the proteolytic enzymes, trypsin and papain, by trying the action of a large number of basic and acidic dyestuffs and also of some narcotics.

Experimental.

It is known that the hydrolytic action of the enzymes, trypsin and papain consists in the breaking up of the $-CO-NH-$ linkage and the amount of hydrolysis was determined by estimating the amino-nitrogen with the micro-Van Slyke apparatus.

The method was to determine the amount of hydrolysis in certain periods produced by a definite weight of the enzyme in a substrate (like peptone) and to compare this with the amount of hydrolysis produced by the same weight of the enzyme after the latter had been exposed to the action of a dyestuff at a concentration of 1/2000 or of a narcotic at a concentration of 1/4000 for an hour. The concentration of the dyestuff was 1/5000 and of the narcotic 1/10,000 in the reaction vessel.

The action of a large number of acidic and basic representative dyestuffs from different series has been tried. The names of the dyes are given in the relevant tables.

The Inhibiting Action of Dyestuffs on Trypsin.

The enzyme material was a preparation of pancreatic trypsin by Messrs. Carnick & Co., U.S.A., that is known by the name of "Trypsogen". Cystin, neutralised and brought up to the desired p_H, was used throughout as an activator (*cf.* Grassman, *Z. physiol. Chem.*, 1930, 186, 183). Phosphate buffer (p_H 8·67) was used in these experiments.

0·2 G. of trypsogen dissolved in water and 0·06 g. of cystin dissolved in a minimum quantity of NaOH solution and brought to the desired p_H (8·67) with HCl solution were transferred to a 25 c.c. measuring flask. 2 C.c. of phosphate buffer of p_H 8·67 and 2 c.c. of the dye solution were also added to the flask. The concentration of the dyestuff in the flask was now 1/2000. The measuring flask was then allowed to stay for an hour in a thermostat at a temperature of 37° so that the enzyme was subjected to the action of dyestuff. After this period 5 c.c. of a peptone solution that was previously brought up to p_H 8·67 were added quickly to the measuring flask and the volume made up to 25 c.c. with water. The concentration of the dyestuff was now 1/5000. 4% Solution of the peptone was used. This peptone solution and water were also kept in the thermostat to acquire the same temperature as the solution, *i.e.*, 37°. Now 2 c.c. of the solution were withdrawn from the flask and allowed to run into the reaction chamber of a micro-Van Slyke apparatus and the NH_2-nitrogen determination made in the usual way (Van Slyke, *J. Biol. Chem.*, 1915, 23, 407). The volume of the nitrogen in the burette was noted. The measuring flask with the solution was kept in the thermostat at the same temperature and a second Van Slyke

determination with 2 c.c. was made after 30 minutes of the first determination. A third determination was made after an hour.

Blank experiment was done without the dyestuff and this blank determination was made from time to time in order to see that the activity of the enzyme remained practically identical and also to take into account the slight changes in activity from time to time. The results obtained are summarised in Table I.

TABLE I.

Percentage inhibition of hydrolysis of peptone with trypsin by different dyestuffs.

$p_H = 8{\cdot}67$. Temp. = 37°.

Basic dyestuffs (1/2000).	% Inhibition.	Basic dyestuffs (1/2000).	% Inhibition.
Brilliant green	18	Toluidine blue	35
Malachite green	0	Janus green	6
Methyl violet	19	Neutral red	0
Crystal violet	0	Chrysoidin	26
Ethyl violet	20	Pyronine	19
Gentian violet	0	Methylene violet	22
Bismarck brown	16	Safranine	0
Methylene blue	0	Auramine	23
Acid dyestuffs (1/2000).	**% Inhibition.**	**Acid dyestuff (1/2000).**	**% Inhibition.**
Erythrosin	26	Soluble blue	16
Eosin yellow	29	Congo red	33
Eosin bluish	28	Benzopurpurin	0
Acid green	20	Orange G.	6
Acid fuchsin	36	Cyanin chloride	11
Water blue	9	Heamotoxylin	30

Conclusion.—It would appear from above that some basic and all acidic dyestuffs are toxic to trypsin at p_H 8·67 and at a concentration of 1/2000. It must be observed, however, that the inhibition never exceeds 36% and in no case reaches 100% that was observed

in the case of the dehydrogenase, fumarase and urease studied by Quastel. It is reasonable to suppose that the inhibiting action of dyestuffs is due to their combination with the enzyme and the enzyme trypsin, therefore, must possess both acidic and basic groups and have amphoteric properties. In this, trypsin resembles fumarase on which both acidic and basic dyestuffs exert toxic action and differs from dehydrogenase and urease whose action is inhibited by basic dyestuffs alone (Quastel, *loc. cit.*).

There is, however, a marked specificity in action both among the acidic and basic dyestuffs and dyestuffs belonging to the same series act in quite different ways. Thus of the basic dyestuffs of the triphenylmethane series, malachite green, crystal violet and gentian violet exert no toxic action, while brilliant green, methyl violet, and ethyl violet inhibit almost to the same extent. The acid dyestuffs of the triphenylmethane series (acid green, acid fuchsin, water blue, soluble blue) are all toxic. Methylene blue is inert while toluidine blue exerts the highest toxic action amongst the basic dyestuffs. Safranine is, as was found by Marston, inert but methylene violet is toxic. Similar behaviour was also observed by Quastel in his investigations, who found, for instance, neutral red inert but Janus green toxic, orange G. inert but congo red highly toxic towards fumarase.

It will thus be seen that the *structure* of the dyestuff molecule is also an important factor in determing its toxic action.

Dyestuffs and Proteolytic Action of Papain.

Papain differs from trypsin in having a p_H optimum ($p_H 5{\cdot}0$) quite different from that of trypsin. The action of all the above dyestuffs was tried on papain. Experiments were done both with Merck's papain (Succus caricae Papayae Siccatus) and also with a sample of papain prepared by us from the fresh milky juice of the carica papaya. This was done by extracting the dried juice with water, centrifuging and precipitating the clear liquid with alcohol. The precipitate was freed from alcohol and was found to be about three times more active than the Merck's preparation.

The activation of the enzyme was affected by cystein, cystin being inert in this case (*vide*, Grassmann, *loc. cit.*). The reaction was carried at p_H 5·0. Gelatine was used as the substrate.

0·2 G. of Merck's papain was taken. A 0·1*M* solution of cystein was prepared and 1 c.c. of the solution was added to the papain solu-

tion for activation. Citrate buffer (p_H 5·0) was used. The cystein solution was brought to the same p_H by adding alkali solution in drops. The solution of papain, cystein (1 c.c.) and the dyestuff (2 c.c.) were taken in a 25 c.c. measuring flask and the mixture was allowed to remain for an hour in the thermostat at 37°. The concentration of the dyestuff was 1/2000. Just before the reaction, 10 c.c. of a 3% gelatine solution at p_H 5·0 was added to the measuring flask and the volume was made up to 25 c.c. with water, the temperature of water and gelatine being also 37°. The concentration of the dyestuff was now 1/5000, 2 c.c. of the mixture were withdrawn and analysed in the Van Slyke apparatus. A second determination was made after an hour of the first reaction. The final determination was made after 2 hours. In the case of laboratory prepared papain 0·08 g. was taken in each reaction. The results obtained are summarised in Table II.

TABLE II.

Percentage inhibition by dyestuffs of gelatine hydrolysis by papain.

p_H=5·0. Temp.=37°.

Basic dyestuff. (1/2000)	% Inhibition Merck's papain.	Prepared papain.	Basic dyestuff. (1/2000)	% Inhibition Merck's papain.	Prepared papain.
Brilliant green	5	6	Toluidine blue	6	4
Malachite green	1	3	Janus green	17	5
Methyl violet	4	4	Neutral red	4	2
Ethyl violet	11	2	Chrysoidin	25	9
Gentian violet	1	6	Pyronin	3	2
Bismarck brown	3	4	Methylene violet	9	4
Methylene blue	6	3	Safranin	1	3
			Auramin	6	2
	Acid dyestuffs (1/2000)				
Erythrosin	26	16	Soluble blue	9	6
Eosin yellow	5	3	Congo red	18	1
Eosine bluish	13	6	Benzopurpurin	13	12
Acid green	1	6	Crystal scarlet	5	16
Acid fuchsin	5	9	Orange G.	12	2
Water blue	2	4	Cyanin chloride	12	6
			Haematoxylin	7	6

The above table makes it clear that as in the case of trypsin both acidic and basic dyestuffs are toxic showing the amphoteric nature of papain. The same specificity of action amongst dyestuffs of the same series is to be observed here. Thus Janus green is fairly toxic while neutral red is inert. Here again the importance of the structure of a dyestuff in determining its toxic action is apparent.

One fact, however, forces itself upon our observation. The dyestuffs appear to be less toxic towards the purer laboratory preparation of papain than towards the less active and therefore, less pure Merck's papain. Similar observations have been made by Quastel (*Biochem J.*, 1932, 26, 1685) in his study of the action of dyestuffs on urease. It is possible that some substance is present in Merck's preparation, which enhances the toxicity of the dyestuffs.

Crystal scarlet alone is more toxic towards the purer preparation. Quastel also found neutral red and Janus green more toxic towards the purer urease.

The Action of Narcotics on the Proteolytic Enzymes, Trypsin and Papain.

Although there are some observations on the effect of narcotics on the Schardinger enzyme and succinic dehydrogenase by Sen (*Biochem. J.*, 1931, 25, 849) and on brain dehydrogenase by Quastel (*ibid.*, 1932, 26, 1672) no data are available on the action of narcotics on proteolytic enzymes. The inhibiting effect of narcotics on enzymic reactions is supposed to be due to the preferential adsorption of these substances on enzyme surfaces and the reaction velocity is diminished owing to the displacement of the substrates from the enzyme surface by the narcotics (Warburg, *Biochem. Z.*, 1921, 119, 134).

The action of the narcotics, diethylurea, ethyl urethane, phenyl urethane, vanillin and also of the following substances, catechol, pyrogallol, gallic acid and sodium hydrosulphite on trypsin and papain has been tried in order to find out if it might throw some light on the nature of the enzymes.

Carnick's "Trypsogen" and papain prepared in the laboratory by us were employed. The same procedure was employed as in the case of the dyestuffs with the difference that the solution of the dyestuff was replaced by the same volume of a solution of the narcotic. The enzyme in presence of the activator was subjected to the action of the narcotic

at a concentration of 1/4000 for an hour and when finally the substrate solution was added and the reaction mixture was made up to 25 c.c., the concentration of the narcotic fell to 1/10,000. The results are as follows.

TABLE III.

Percentage inhibition by narcotics of peptone hydrolysis by trypsin.

p_H=8·67. Temp.=37°

Narcotic $\left(\frac{1}{4000}\right)$	Percentage inhibition after 35 min.	65 min.
Pyrogallol	2	9
Ethyl urethane	0	3
Diethylurea	0	9
Vanillin	6	7
Gallic acid	0	10
Catechol	0	10
Phenyl urethane	0	0
Sodium hydrosulphite	0	7

TABLE IV.

Percentage inhibition by narcotics of gelatine hydrolysis by papain.

p_H=5. Temp.=37°.

Narcotic $\left(\frac{1}{4000}\right)$	Percentage inhibition after 60 min.	120 min.
Pyrogallol	0	0
Ethyl urethane	5	5
Diethyl urea	0	0
Vanillin	0	9
Gallic acid	5	16
Catechol	0	0
Phenyl urethane	3	3
Sodium hydrosulphite	0	0

It will be observed that in the case of papain the narcotics are without any action, only gallic acid, vanillin and ethyl urethane causing some inhibition. The inhibiting action increases with time. In the case of trypsogen practically all the substances tried show no effect for the first hour of the reaction but an appreciable inhibiting action is to be noticed after two hours. This shows that the adsorption of the narcotics on trypsin surface is very slow and is inappreciable for the first two hours. Similar results were obtained by Quastel in the case of the action of Somnifaine on brain dehydrogenase.

Our grateful thanks are due to Prof. J. C. Ghosh for his kind interest in this investigation.

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Received November 17, 1934.

Naturwissenschaften, 46:206 (1959)

Partial and Temporary Methemoglobin Formation by Deoxygenation of Oxyhemoglobin with Sodium Dithionite*

Sodium dithionite ($Na_2S_2O_4$) has been used successfully in experimental work to deoxygenate or to reduce blood pigment. One can easily follow the effect photometrically in the red wavelengths because Hb exhibits a higher than does O_2Hb. In testing for the minimum concentration of $Na_2S_2O_4$ which was optimum for the deoxygenation of an O_2Hb solution, we were astonished to find that first, as expected, the light absorption (using Filter OG 3, Schott) increased as the $Na_2S_2O_4$ concentration increased, but after a certain concentration ("intermediate concentration") was exceeded, the amount of light absorption decreased (Fig. 1). At exactly three times the value of the "intermediate concentration," the amount of absorption did not change any further ("end concentration"). Spectrophotometry of the blood pigment at the "end concentration" shows a typical absorption curve of oxygen-free (reduced) Hb. The absorption curve of the blood pigment at the "intermediate concentration" on the other hand, showed a smooth maximum between 640 and 620 mμ (Fig. 2), and a lower maximum at 560 mμ. The suspicion that the higher extinction of the "intermediate concentration" in the red region resulted from the presence of some methemoglobin, was given a basis in the loss of the high extinction when KCN was added. An approximate calculation showed that perhaps a quarter of the pigment was methemoglobin (See Fig. 2). With the $Na_2S_2O_4$ concentration

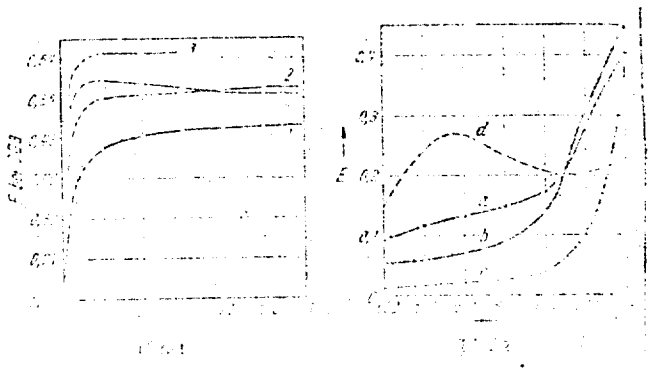

Fig. 1. Curve showing the increase in light absorption using filter OG 3, the effect of $Na_2S_2O_4$ at pH=6.8 (phosphate buffer) on oxyhemoglobin. Blood pigment concentration = 970 mg/100 ml; $Na_2S_2O_4$ concentration: 1 = 14.6 mg/100 ml, 2 = 19.2 mg/100 ml, 3 = 22.3 mg/ 100 ml, 4 = 42.7 mg/100 ml.

Fig. 2. Absorption spectra of a blood pigment solution of 100 mg/ 100 ml at pH = 6.8, the effect of 11 mg/100 ml (a) and 25 mg/100 ml (b) $Na_2S_2O_4$ respectively. For comparison the absorption curves of oxyhemoglobin (c) and methemoglobin (d) are given.

below the "intermediate concentration," the absorption curve exhibits a mixture of O_2Hb, Hb and methemoglobin.

Below and at the "intermediate concentration," the transient products controlled the reaction, proceeding to the final value quickly and smoothly. Above the "intermediate concentration," the products are noteworthy in that the absorption reached a high value for a short time, and then within about 60 seconds, sank to a final value (Fig. 1, Num. 4). If one increased the $Na_2S_2O_4$ concentration further, the peak came, with respect to the above, lower and more quickly. One may assume that the transient increase in absorption corresponded to a temporary increase in methemoglobin production.

Conclusions. In measurements of oxygen-dissociation of O_2Hb by the addition of $Na_2S_2O_4$, the production of methemoglobin must be seriously considered in the calculations. That $Na_2S_2O_4$ in the presence of O_2 promotes the oxidation of Hb to methemoglobin has already been described in 1924 by Conant and Fieser[1].

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Received on January 8, 1959

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*) With the support of the German Reserach Society

1) Conant, J. R. and L. F. Fieser, J. Biol. Chem. 62:623 (1924)

Naturwissenschaften, 46: 206 (1959)

absorption aufweist als O_2Hb. Beim Austesten der Minimalmenge an $Na_2S_2O_4$, die für die Desoxygenierung einer O_2Hb-Lösung erforderlich ist, fiel uns auf, daß zunächst wie erwartet mit steigender $Na_2S_2O_4$-Konzentration auch die Lichtabsorption (bei Filter OG 3, Schott) anstieg, daß sie aber nach Überschreiten einer gewissen Konzentration („Zwischenkonzentration") wieder abfiel (Fig. 1). Vom knapp dreifachen Wert der „Zwischenkonzentration" an veränderte sich dann die Absorption nicht mehr weiter („Endkonzentration"). Spektralphotometrisch zeigte der Blutfarbstoff bei „Endkonzentration" die typische Absorptionskurve von sauerstofffreiem (reduziertem) Hb. Die Absorptionskurve des Blutfarbstoffs bei „Zwischenkonzentration" wies demgegenüber ein flaches Maximum zwischen 640 und 620 mμ auf (Fig. 2) und ein niedrigeres Maximum bei 560 mμ. Der Verdacht, daß die höhere Extinktion im roten Bereich bei „Zwischenkonzentration" durch einen Anteil an Methämoglobin bedingt sei, ließ sich dadurch stützen, daß Zusatz von $K_3Fe(CN)_6$ die Extinktionserhöhung beseitigte. Eine Überschlagsrechnung ergibt,

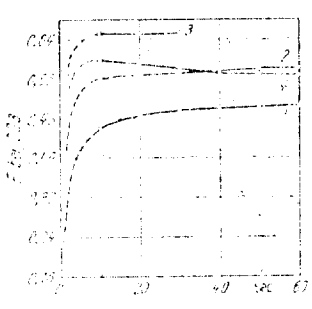

Fig. 1

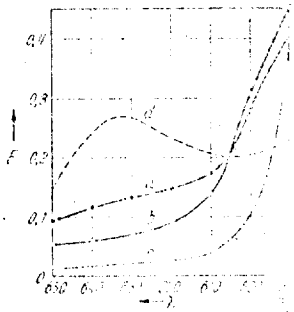

Fig. 2

Fig. 1. Ablauf des Anstiegs der Lichtabsorption bei Filter OG 3 nach Einwirkung von $Na_2S_2O_4$ bei p_H 6,8 (Phosphatpuffer) auf Oxyhämoglobin. Blutfarbstoffkonzentration = 970 mg/100 ml und $Na_2S_2O_4$-Konzentration: 1 = 14,0 mg/100 ml, 2 = 18,2 mg/100 ml, 3 = 22,3 mg/100 ml, 4 = 42,7 mg/100 ml

Fig. 2. Spektrale Absorption einer Blutfarbstofflösung von 100 mg/100 ml bei p_H 6,8, Einwirkung von 14 mg/100 ml für bzw. 25 mg/100 ml (b) $Na_2S_2O_4$. Zum Vergleich ist die Absorptionskurve von Oxyhämoglobin (c) und von Methämoglobin (d) eingezeichnet

daß bei „Zwischenkonzentration" etwa ein Viertel des Farbstoffs als Methämoglobin vorliegt (vgl. Fig. 2). Bei $Na_2S_2O_4$-Mengen unterhalb der „Zwischenkonzentration" lag nach den Absorptionskurven ein Gemisch von O_2Hb, Hb und Methämoglobin vor.

Im zeitlichen Ablauf steuert die Reaktion unterhalb und bei „Zwischenkonzentration" rasch und gleichmäßig ihrem Endwert zu. Oberhalb der „Zwischenkonzentration" ist der Ablauf dadurch bemerkenswert, daß die Absorption kurzfristig einen höheren Wert erreicht und dann innerhalb von etwa 60 sec auf den endgültigen Wert absinkt (Fig. 1, Ziff. 3). Steigert man die $Na_2S_2O_4$-Menge weiter, wird der Ausschlag nach oben geringer und kürzer. Man wird annehmen dürfen, daß der vorübergehenden Absorptionssteigerung eine vorübergehende vermehrte Methämoglobinbildung entspricht.

Nachfolgerung. Bei Messung der Sauerstoffdissoziation mit Hilfe von $Na_2S_2O_4$ muß eine Methämoglobinbildung als Störfaktor in Rechnung gesetzt werden. Daß $Na_2S_2O_4$ bei Anwesenheit von O_2 die Oxydation von Hb zu Methämoglobin begünstigt, ist bereits 1924 von CONANT und FIESER[1] beschrieben worden.

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KLAUS BETKE und PETER SCHOLZ

Eingegangen am 8. Januar 1959

p. 206

Partielle und vorübergehende Methämoglobinbildung bei Desoxygenierung von Oxyhämoglobin durch Natriumdithionit[*])

Natriumdithionit ($Na_2S_2O_4$) wird in experimentellen Arbeiten gern benutzt, um Blutfarbstoff zu desoxygenieren oder zu reduzieren. Man kann dann Filter im roten Wellenbereich spektrometrisch gut auswerten, weil Hb dort eine höhere Lichtab-

*) Mit Unterstützung der Deutschen Forschungsgemeinschaft.

[1]) CONANT, J. B., u. L. F. FIESER: J. Biol. Chem. 62, 625 (1924).

SODIUM HYDROSULPHITE IN TREATMENT OF ACUTE ARSENICAL POISONING*

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Acute arsenical poisoning is still a problem of vital interest to the clinician. Although the number of cases of accidental poisoning has been considerably reduced by virtue of certain restrictions that have been placed against the industrial use of the substance, and the dissemination of knowledge regarding its toxic properties, cases of acute intoxication are not infrequent. In the search for suitable antidotes with which to combat the toxic manifestations of an orally administered arsenical compound, many substances have been introduced.

Unfortunately these so-called arsenic antidotes have not found support in animal experimentation, in which it becomes possible to draw satisfactory conclusions as to the efficacy of the preparation by establishing conditions of uniformity in dosage and treatment, as well as the employment of an adequate number of control animals.

In a recent paper from this laboratory[1] it was conclusively shown that sodium thiosulphate was valueless in the treatment of poisoning from the oral administration of potassium arsenite. Here the fatal outcome was not even retarded, although the supposed antidote was given immediately following the administration of a minimal lethal dose of the arsenic preparation.

Our interest in this work has been revived by the investigations of Hesse,[2] who reports that he has employed sodium hydrosulphite with success in the treatment of experimental mercuric chloride poisoning. The validity of Hesse's observations has been attested to some extent in this laboratory by Haskell and his associates, who have found that sodium hydrosulphite if given immediately following the oral administration of a fatal dose of mercuric chloride will, in the majority of instances, save the animal.

The use of sodium hydrosulphite in mercuric chloride poisoning has led us to conduct a series of experiments to determine whether the compound would exert any favorable influence in cases of acute arsenic poisoning. These experiments have been carried out on apparently healthy dogs following a twenty-four hour period of starvation, so that the presence of food in the stomach would not influence absorption of the arsenic preparation. To prevent vomiting, 10 mg. of morphine sulphate per kilogram was injected subcutaneously, followed in half an hour by the oral administration of the poison. As a source of arsenic we have employed solution of potassium arsenite-U. S. P. (Fowler's solution), which was administered from a buret into a funnel connected with the stomach tube, and rinsed down with 25 cc. of tap water. From previous experiments it was found that the minimal lethal dose of solution of potassium arsenite was between 0.5 and 0.75 cc. per kilogram of body weight, when given orally to dogs. The mortality of a series of eighteen dogs given 0.75 cc. per kilogram was 100 per cent, and that of thirteen dogs given a dose of 0.5 cc. per kilogram, 46.1 per cent.

In our first experiment, however, we employed 1 cc. of solution of potassium arsenite per kilogram orally, which was immediately followed by the sodium hydrosulphite, 100 mg. per kilogram in the form of a 10 per cent solution, and was rinsed down with 25 cc. of normal hydrochloric acid. The results are shown in table 1.

Sodium hydrosulphite must not be confused with the thiosulphate ($Na_2S_2O_3$), which is sometimes erroneously called hyposulphite. The compound with which the investigation reported in this paper has been conducted has the formula $Na_2S_2O_4$. It is very soluble in water, with the evolution of heat and the liberation of sulphur dioxide. With solutions of potassium arsenite a dark brown precipitate of the subsulphide of arsenic is formed, the reaction being accelerated by the addition of dilute mineral acids. The toxicity of sodium hydrosulphite is apparently low; we have given to dogs as much as 1 Gm. per kilogram of body weight without any apparent injurious effect. However, the compound possesses one objectionable feature in that it induces vomiting in many instances in spite of the previous administration of morphine. For this reason Hesse advises alkalization of the solution of sodium hydrosulphite, which not only increases the stability of the solution but renders it less irritant to the gastric mucosa. As rational as this procedure would seem, it is no safeguard against emesis. Since the alkalized solution does not produce any immediate visible reaction with potassium arsenite there seems to be no advantage in such a procedure; consequently we felt that it would be a better plan to administer a freshly prepared solution of sodium hydrosulphite and immediately follow it with 25 cc. of normal hydrochloric acid. Under these conditions the compound would be fairly rapidly decomposed but certainly more effective in precipitating the arsenic. After the administration of the antidote and acid solutions, it was customary to observe the animals for a period of half an hour and to reject those animals in which vomiting occurred.

TABLE 1.—*Results Following the Immediate Use of Sodium Hydrosulphite in Poisoning from the Oral Administration of Potassium Arsenite Solution*

Weight of Dog, Kg.	Solution of Potassium Arsenite-U. S. P., Cc. per Kg.	Sodium Hydrosulphite, Mg. per Kg.	Result
10.1	1	100	Survived*
7.27	1	0	Died 8 hours
5.0	1	100	Survived*
6.6	1	0	Died 9 hours

* If animal was living at the end of two weeks, it was reported as survived.

It is quite obvious from table 1 that the presence of the sodium hydrosulphite has exerted a most favorable influence, since both treated animals survived, and the untreated ones died within twenty-four hours. This is, however, of little practical importance from a clinical standpoint, as in most cases of clinical arsenic poisoning there is a considerable lapse of time before the patient comes under observation. The experiment may be of value in showing that inorganic arsenic compounds are rendered less toxic by the administration of sodium hydrosulphite.

The next experiments were conducted with the view of determining the period of effectiveness for the antidote by allowing varying periods of time, ranging from three to thirty minutes, to elapse before the hydrosulphite was administered. The condensed protocols are shown in table 2.

* From the Department of Pharmacology, Medical College of Virginia.

1. Haag, H. B., and Bond, W. R.: Value of Sodium Thiosulphate in Poisoning from Oral Administration of Arsenic, J. A. M. A. 89:1219 (Oct. 15) 1927.

2. Hesse, Erich: Versuche zur Therapie der Quecksilbervergiftung, Arch. f. exper. Path. u. Pharmakol. **107**:43, 1925.

The results obtained in this experiment, while somewhat discouraging, plainly show the necessity of immediate treatment, as well as the rapidity with which potassium arsenite is absorbed from the stomach. The mortality of the treated animals was 100 per cent when

TABLE 2.—*Results Following the Use of Sodium Hydrosulphite in the Treatment of Dogs Poisoned by the Oral Administration of Potassium Arsenite Solution*

Weight, Kg.	Arsenic Solution, Cc. per Kg.	Sodium Hydrosulphite, Mg. per Kg.	Time Interval in Minutes	Result 24 Hours
13.2	1	None	..	Dead
12.4	1	None	..	Dead
10.8	1	None	..	Dead
4.57	1	None	..	Dead
14.0	1	100	30	Dead
12.5	1	100	30	Dead
5.0	1	100	30	Dead
7.45	1	100	30	Dead
14	1	100	4.75	6 days
9.1	1	100	4.25	Survived
10.8	1	100	3	Survived
8.55	1	100	3	Survived
10.2	1	100	10	Dead
11.7	1	100	10	Dead
13.7	1	100	10	Dead
11.4	1	100	0.25	Dead

the lapse of time was greater than five minutes. All animals treated before this time survived, with the exception of one which died on the sixth day without developing the usual symptoms of acute arsenic poisoning.

Heretofore we had been employing doses of arsenic approximately twice the minimal fatal dose. In the next experiment it was thought advisable to reduce the quantity of arsenic solution to 0.75 cc. per kilogram and observe the effect of this reduction in dosage on the period of effective treatment. It may be readily seen from table 3 that less than 10 per cent of the animals treated with sodium hydrosulphite within ten minutes after the administration of a fatal dose of

TABLE 3.—*Results Following the Use of Sodium Hydrosulphite in the Treatment of Poisoning from the Oral Administration of Potassium Arsenite Solution-U. S. P., 0.75 Cc. per Kilogram of Body Weight (Dogs)*

Weight, Kg.	Arsenic Solution, Cc. per Kg.	Sodium Hydrosulphite, Mg. per Kg.	Time Interval in Minutes	Results
4.6	0.75	None	..	Died 18 hours
3.86	0.75	None	..	Died 18 hours
3.8	0.75	None	..	Died 21 hours
6.55	0.75	None	..	Died 24 hours
8.9	0.75	None	..	Died 24 hours
10.9	0.75	None	..	Died 24 hours
11.6	0.75	None	..	Died 24 hours
13.7	0.75	None	..	Died 24 hours
7.7	0.75	100	5	Died 56 hours
6.1	0.75	100	5.75	Survived
13.65	0.75	100	6	Survived
10.3	0.75	100	6	Survived
8.97	0.75	100	6	Survived
7.7	0.75	100	6	Survived
8.5	0.75	100	10	Survived
8.1	0.75	100	10	Survived
6.9	0.75	100	10	Survived
8.3	0.75	100	10	Survived
8.18	0.75	100	10	Survived
7.7	0.75	100	15	Died 24 hours
7.0	0.75	100	15	Survived
8.5	0.75	100	16	Died 24 hours
5.8	0.75	100	18	Died 24 hours
7.65	0.75	100	20	Died 18 hours
8.1	0.75	100	20	Died 24 hours
6.7	0.75	100	11	Survived

arsenic died. The mortality of the control animals and those treated after this time was 100 per cent, the greatest prolongation of life being only forty-eight hours.

It should be borne in mind that these experiments have been conducted under such conditions as would markedly facilitate the absorption of the arsenic preparation. It is not surprising, therefore, that the antidote was ineffective after the lapse of ten minutes, since a fatal dose of arsenic had already been absorbed, and at this stage it is extremely doubtful whether lavage would accomplish the desired result. The presence of food in the stomach, as would ordinarily be the case clinically, would undoubtedly delay absorption of the poison to such an extent that the period of effective treatment would be much prolonged.

SUMMARY

We do not wish to recommend the use of sodium hydrosulphite in the treatment of acute arsenic poisoning as a measure intended to supplant gastric lavage, the importance of which is unquestioned in poisoning from any orally administered drug. As an adjunct to the latter, however, the compound may prove quite effective, particularly in those cases in which the presence of undigested food in the stomach might embarrass the progress of lavage. The immediate administration of appropriate amounts of sodium hydrosulphite may serve to fix or render unabsorbable the arsenic until thorough lavage can be effected, if not to be solely responsible for the patient's recovery.

Science, 103 (2674): 399-401, 1946

Interference With the Antibacterial Action of Streptomycin by Reducing Agents[1]

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The authors have repeatedly observed that the antibacterial action of streptomycin is significantly reduced by incubation in an anaerobic jar. When filter-paper discs are saturated with streptomycin and placed on the surface of infusion blood agar plates inoculated with *E. coli*, the resulting zones of inhibition on plates incubated anaerobically are much smaller than those on the comparable aerobic plates. The reduced activity of streptomycin anaerobically is not due to better growth conditions, since *E. coli* produces its maximum growth when incubated aerobically. Similar results may be obtained with staphylococci and other species of bacteria whose growth is favored by aerobic incubation.

These observations seemed to us to be significant, and further studies were carried out, the results of which suggest that they may have some bearing on the antibacterial action of streptomycin.

Method and results. Duplicate sets of infusion agar plates containing 2-fold concentrations of streptomycin were inoculated with various species of bacteria. One set of plates was incubated aerobically, the other in the anaerobic jar by the palladium-hydrogen method as modified by Spaulding and Goode (5). Plates were read for evidence of growth inhibition at the end of 24 and 48 hours incubation at 37° C. Table 1 shows the results of these tests.

TABLE 1

INHIBITION OF BACTERIAL GROWTH BY STREPTOMYCIN ON INFUSION AGAR

Organism	Incubation	Growth* in presence of streptomycin; units/ml. of agar							
		None	1.0	2.0	4.0	8.0	16.0	32.0	64.0
E. coli-S	aerobic	4	4	1	–	–	–	–	–
	anaerobic	3	3	3	3	3	3	–	–
E. coli-M	aerobic	4	4	2	–	–	–	–	–
	anaerobic	3	3	3	2	–	–	–	–
S. aureus-SM	aerobic	4	4	1	1	1	–	–	–
	anaerobic	4	4	3	3	2	2	–	–
S. aureus #7739	aerobic	4	1	–	–	–	–	–	–
	anaerobic	3	3	3	3	2	1	–	–
S. paratyphi B	aerobic	4	4	4	3	–	–	–	–
	anaerobic	3	3	3	3	2	2	2	1
S. typhi-murium	aerobic	4	4	4	3	2	–	–	–
	anaerobic	2	2	2	2	2	2	1	–
Pneumo. Type I	aerobic	4	4	4	4	4	1	–	–
	anaerobic	4	4	4	4	2	1	–	–
Str. Hemolyticus C-203	aerobic	4	4	4	4	4	3	–	–
	anaerobic	4	4	4	4	3	1	–	–

* Readings made at end of 24 hours incubation; 48-hour readings were essentially the same.

Under anaerobic conditions the amount of streptomycin necessary to produce bacteriostasis was from 2 to 16 times greater than the comparable aerobic concentration. With one exception (*S. aureus*-SM), these results were observed only with organisms which produced their maximum growth on aerobic plates.

A similar experiment was carried out with extract agar. Although there was some evidence that the antibacterial action of streptomycin was greater on the aerobic plates, the difference was not large. It appeared that various reducing agents in the infusion medium played a role in inhibiting streptomycin anaerobically. The greater reducing power of infusion agar was evident from the observation that methylene blue in a concentration of 1:100,000 in this medium

[1] Streptomycin used in this study was generously supplied through the courtesy of Dr. D. F. Robertson, Merck and Company, Inc., Rahway, New Jersey.

was reduced during anaerobic incubation, while methylene blue extract agar plates similarly incubated were not reduced. The presence or absence of streptomycin had no apparent effect.

The anti *E. coli* action of streptomycin was subsequently determined in tryptone and infusion broth containing cysteine or sodium thioglycollate (Table 2).

TABLE 2

THE EFFECT OF CYSTEINE AND THIOGLYCOLLATE ON THE ANTIBACTERIAL ACTION OF STREPTOMYCIN AGAINST *E. coli*

Medium	Inhibiting concentration of streptomycin Units/ml.
Tryptone*	1.0
Tryptone plus 0.1 per cent thioglycollate	2.0
Tryptone plus 0.1 per cent cysteine	64.0
Infusion†	16.0
Infusion plus 0.1 per cent thioglycollate	64.0
Infusion plus 0.1 per cent cysteine	> 128.0

* Difco tryptone 1.0 per cent.
† Veal infusion, 1.0 per cent tryptose, 0.03 per cent glucose.

Aside from the added SH compounds, infusion broth itself contains interfering substances, since 16 times as much streptomycin was necessary for bacteriostasis in the infusion control as in the tryptone control. The presence of cysteine increased the concentration required for bacteriostasis 64-fold in tryptone and at least 8-fold in infusion broth; the increase with thioglycollate was only 2- to 4-fold.

TABLE 3

EFFECT OF INORGANIC REDUCING AGENTS ON THE ANTIBACTERIAL ACTION OF STREPTOMYCIN AGAINST *E. coli*

Reducing agent*	Inhibiting concentration of streptomycin Units/ml.
Tryptone control	2.0
0.5 per cent sodium formate	32.0
0.05 per cent stannous chloride	32.0
0.05 per cent sodium bisulfite	32.0
0.05 per cent sodium hydrosulfite	16.0
0.5 per cent sodium thiosulfate	32.0

* Final concentration in tryptone broth.

The effect of cysteine on streptomycin could be due either to a reducing action or to some specific chemical reaction, especially with the sulfhydryl groups. The inactivation of penicillin (*2*) and other antibiotic agents (*1*) by cysteine is well known. The action of cysteine on streptomycin appears, however, to be different from that on penicillin, as was demonstrated in the recent report of Denkelwater, Cook, and Tishler (*4*), published while this manuscript was being prepared. These workers state that the inactivation of streptomycin by cysteine is reversible. Similar interference by sulfhydryl compounds with the antibacterial properties of mercurials (*6*) and quinones (*3*) has been reported.

If interference with the activity of streptomycin by sulfhydryl compounds is due to reducing powers, other reducing agents should likewise interfere with its action. Consequently, various reducing agents have been tested. Each of the substances studied definitely antagonized the antibacterial action of streptomycin against *E. coli,* as shown in Table 3. It is evident that the antibacterial action of streptomycin is greatly diminished by a variety of reducing agents other than sulfhydryl compounds.

Discussion. Reducing agents definitely antagonize the antibacterial activity of streptomycin. Further work will be required, however, to determine the true nature of this interference. Whether it is due to a lowering of oxygen tension or to some specific chemical reaction or both acting simultaneously, is still unknown. The SH compounds and the inorganic reducing agents could function in either capacity. The reduction in activity of streptomycin in the presence of glucose, as reported by Waksman and co-workers (*9*), could be attributed to the reducing action of this carbohydrate.

It is entirely possible that this phenomenon is related to the mode of action of streptomycin. This antibiotic displays its greatest activity against bacteria which grow better aerobically than anaerobically, e. g. *E. coli, S. aureus,* and *Myco. tuberculosis.* None of the obligate anaerobes is susceptible (*7*). The antibacterial action of streptomycin may be due to its ability to block some enzyme system, oxidative in nature, which is essential only to the growth of susceptible aerobic bacteria, an enzyme system which anaerobes do not possess. If this is true, it is possible that the antagonism by the reducing agent is a specific effect upon this streptomycin-enzyme relationship rather than a direct effect on the streptomycin itself. In fact, addition of the SH compounds and the inorganic reducing agents to a concentrated solution of streptomycin (1,000 units/ml.) does not result in material loss of activity, although Denkelwater and co-workers (*4*) recently reported that this antibiotic in a lower concentration was inactivated by cysteine.

Certain other practical implications are worthy of note. It is conceivable that in certain parts of the body where organic reducing agents are present or where a low oxygen tension exists, larger concentrations of streptomycin may be necessary to inhibit the growth of bacteria. Elias and Durso (*5*) recently reported that typhoid bacilli were isolated from stools in spite of the presence of large concentrations of streptomycin. These investigators suggested the presence in the body of a substance inhibitory to strepto-

mycin. It remains to be seen whether these two phenomena are related.

It is evident that the choice of a medium is important in assaying streptomycin and in determining the susceptibility of bacteria to its antibacterial activity. Because of its antagonism for streptomycin, an infusion medium would be an unwise choice for this purpose. Eventually one of these reducing agents may prove helpful in testing the sterility of streptomycin solutions or in the culturing of fluids from patients under treatment. Cysteine may prove to be such an agent.

Summary. The antibacterial activity of streptomycin in infusion agar plate cultures of *E. coli* and other bacteria is diminished by anaerobic incubation. The bacteriostatic activity of this antibiotic for *E. coli* is reduced in the presence of cysteine, sodium thioglycollate, stannous chloride, sodium bisulfite, sodium hydrosulfite, sodium formate, and sodium thiosulfate. Cysteine was the most active of the agents tested. Further investigation is necessary to determine the nature of this interference. It is possible that this phenomenon is related to the mode of action of streptomycin.

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Inhibition of Prothrombin, Thrombin and Autoprothrombin C with Enzyme Inhibitors*

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As a means of determining active groups on a molecule organic chemists use reagents which specifically react with chemical groups. Biochemists have employed this same method to determine active sites on enzymes. Using these methods the inhibitory effect of various chemical modifications of three molecules important in blood coagulation, prothrombin, thrombin and autoprothrombin C, was explored in this investigation.

Bovine prothrombin, thrombin and autoprothrombin C have been isolated in this laboratory, and their amino acid composition determined. For easy reference to the amino acid residues of all three, a table has been published by Seegers, Cole, Harmison and Marciniak (21). All amino acids are present in each of these three molecules, and hence all of the polar groups – amino, imidazole, guanidyl, carboxyl, disulfide, sulfhydryl, phenol, hydroxyl, indole, amide, and thiomethyl – are present, and could function as an active site.

By specifically blocking groups and then measuring the activity of the molecule to see if this produced a loss, we expected to get information concerning molecular structures essential for activity. If activity was lost, one might assume that this particular group was essential for the molecule to display its proper activity. It is possible that more than one group is essential for the activity, but unfortunately the number of specific blocking reagents is very limited. So, in all cases, several reagents were used to determine the essential position of one particular group.

Nanninga (15) studied the effect of numerous chemicals on the activity of thrombin and concluded that thrombin has an essential amino group. He found neither sulfhydryl groups nor disulfide bonds essential for thrombin activity.

* This investigation was supported in part by a research grant HE 03424-08 from the National Heart Institute, National Institutes of Health, U.S. Public Health Service.

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Carter and Warner (4, 5) found that reduction of disulfide bonds resulted in decreased activity in prothrombin but not thrombin. Sulfhydryl blocking agents did not result in lost activity of either prothrombin or thrombin. They concluded from these observations that prothrombin has an essential disulfide bonding, but thrombin does not, and that sulfhydryl groups are not essential for the activity of either.

Koppel, *et al.* (8, 9), found that sulfhydryl blocking agents decreased the prothrombin concentration of plasma as determined by the two-stage analysis. Through a series of substitution experiments which included the substitution of purified or concentrated clotting factors, they concluded that the essential sulfhydryl group was not in prothrombin. It was an accelerator of prothrombin activation, which they decided was Ac-globulin (factor V). Aoki, Harmison and Seegers (1) confirmed this observations. They found that their highly concentrated preparations of Ac-globulin (factor V) lost activity following incubation with sulfhydryl blocking agents.

Materials and Methods

Bovine prothrombin was prepared according to the method of Seegers (19, 24). Thrombin and autoprothrombin C were prepared by methods previously described (21). The chemicals used were added to distilled water immediately prior to use. The pH of each solution was neutralized so that it was between pH 6.5 and 7.5, and then made up to the desired molarity, which in most cases was 0.1 M. An aliquot of this solution was diluted so that two different concentrations of each chemical were used. Unless otherwise indicated, pH adjustments of acidic solutions were accomplished with NaOH, and of alkaline solutions with HCl.

Each chemical was tested against the three proteins, prothrombin, thrombin and autoprothrombin C. One part chemical and one part protein to be tested was mixed together in a plastic tube, making the final concentration of chemical in most cases 0.05 and 0.01 M. At zero time, immediately upon mixing protein and chemical, an aliquot was removed and its activity determined. This value was considered to be 100% activity, and also served as a control to determine if at this concentration the chemical reagent interfered with the assay procedure for the protein. The mixture of one part chemical reagent and one part protein was left in stoppered plastic tubes at room temperature. After two hours another aliquot was removed and the activity of the protein again measured. After 24 hours a third aliquot was taken and its activity determined. The protein activities after two and 24 hours incubation were reported as percentages of the activities at zero time.

Controls were set up which contained one part of the protein and one part distilled water. These controls measured any spontaneous deterioration of the protein during the 24-hour incubation period. The proteins were in concentrated solutions (15,000 U/ml prothrombin or thrombin and 1,000 U/ml autoprothrombin C) during incubation periods and could thus be greatly diluted before assaying their activities. The chemicals with which they had been incubated were, consequently, also diluted. This was perhaps sometimes important in preventing the chemical from interfering with the assay.

The prothrombin concentrations were determined by the modified two stage assay of Warner and Seegers (26). Thrombin concentration was determined by its ability to clot fibrinogen as described by Seegers and Smith (26). The method described by Cole, Marciniak and Seegers (8) was employed to quantitate autoprothrombin C activity.

Experimental Results

Table 1 is designed as a summary of the results with numerous chemicals which were tested.

Reducing agents. Reducing agents act on unmodified proteins to convert disulfide bonds to sulfhydryl groups. The reducing agents which were employed in these experiments included several reagents having SH groups: cysteine, thioglycolic acid, 2, 3-dimercapto-1-propanol (BAL) and reduced glutathione. Other reducing agents, having no SH group, included: sodium sulfite, sodium bisulfite, sodium hydrosulfite, and ascorbic acid.

In this study all of the reducing agents altered the prothrombin and autoprothrombin C molecules so that they no longer displayed their typical activity. The thrombin molecule was unaffected by the reducing agents having SH groups. It was somewhat affected by sodium sulfite in two hours; however 24 hours were required to show a marked inhibitory effect of sodium sulfite, sodium bisulfite, and sodium hydrosulfite upon the ability of thrombin to clot fibrinogen.

Later we found that thrombin could after all be inactivated with reducing agents. Thioglycolic acid, first neutralized with sodium hydroxide, destroyed the activity at 0.2 M concentration. At 0.05 M some of the activity was first lost and then recovered spontaneously. Since much depends upon the relative concentration of thrombin and reducing agent we at first failed to observe a change.

Oxidizing agents. Oxidizing reagents containing the disulfide linkage specifically oxidize proteins having free sulfhydryl groups. Typical reagents which were used for studying this group were: cystine, and oxidized glutathione. As a strong oxidizing agent potassium permanganate was employed. The mild oxidizing agents employed were: hydrogen peroxide, potassium ferricyanide, 2,6-dichlorophenolindophenol sodium salt, and sodium selenite.

Oxidizing reagents having disulfide linkages had no inhibitory effect on prothrombin, thrombin or autoprothrombin C activity. A strong oxidizing agent such as potassium permanganate, even in low concentration, inhibited all three molecules within two hours incubation. With mild oxidizing agents there was decreased activity of all three after 24 hours, but varied in their inhibitory effect in two hours, generally showing a low degree of inactivation of prothrombin, thrombin and autoprothrombin C. Autoprothrombin C was the most sensitive of the three to hydrogen peroxide. Its activity was reduced to approxi-

Table 1. Chemical Modification of Prothrombin, Thrombin and Autoprothrombin C and Effect of Their Activity.

Reagent	Prothrombin		Thrombin		Autoprothrombin C	
	2 hrs	24 hrs	2 hrs	24 hrs	2 hrs	24 hrs
Reducing agents:						
Ascorbic acid			0	0		
Thioglycolic acid			0	0		
BAL			0	0		
Reduced glutathione			0	0		
Cysteine			0	0		
Sodium bisulfite						
Sodium hydrosulfite			0			
Sodium sulfite						
Alkylating agents:						
Iodoacetate 0.05 M	0					
Iodoacetate 0.01 M	0		0	0	0	0
Oxidizing agents:						
Oxidized glutathione	0	0	0	0	0	0
Cystine	0	0	0	0	0	0
Hydrogen peroxide	0		0			
Potassium permanganate[1]						
Potassium ferricyanide	0		0	0	0	
2,6-Dichlorophenolindophenol[2]			0			
Sodium selenite	0	0	0	0	0	0
Mercaptide forming agents:						
Parachloromercuribenzoate[3]	0	0	0	0	0	0
Mapharside	0	0	0	0	0	0
Merthiolate	0	0	0	0	0	0
Phenylmercuric Hydroxide	0		0	0	0	0
P-arsanilic acid	0	0				
Phenylmercuric nitrate	0		0	0	0	0
Carbonyl reagents:						
Phenylhydrazine						
Hydroxylamine			0	0		
Sodium bisulfite						
Amino group reagents:						
Formaldehyde[4]					0	
S-acetylmercaptosuccinic anhydride					0	

1) Final concentration 0.0005 M.
2) Final concentration 0.005 M.
3) Final concentration 0.003 M.
4) Final concentration 0.015 M.

Tab. 1: Continued

Reagent	Prothrombin		Thrombin		Autoprothrombin C	
	2 hrs	24 hrs	2 hrs	24 hrs	2 hrs	24 hrs
Miscellaneous:						
Phenol[5]	0	0	0	0	0	0
Guanidine hydrochloride	0	0	0	0	0	0
Lithium bromide	0	0	0	0	0	0
Potassium ferrocyanide	0				0	

5) Final concentration 1% solution.

The first column under each protein tabulates the effect of two hours incubation with the chemical, and the second column 24 hours incubation. If from 90 to 100% of the original activity was present at the end of the time period, it is designated in the table by zero. One plus indicates that between 70 and 90% of the original activity remained. Two plus indicates that between 50 and 75% of the original activity was retained. Three plus indicates that between 25 and 50% of the original activity was present, and four plus indicates that less than 24% of the original activity remained at the end of the period of incubation. Similar results were obtained whether the final concentration of the chemical was 0.05 or 0.01 M except in the case of iodoacetate. Therefore results for both concentrations of iodoacetate are recorded in the table. Other results in the table are for a final concentration of 0.05 M unless otherwise indicated. Solutions were neutralized to a pH range of pH 6.5 to 7.5. Incubation was carried out at room temperature. In the case of thrombin reacting agents such as thioglycolic acid are effective in higher concentrations.

mately 60% of its original activity within the first two hours whereas the others still retained their activity. All of the materials, however, had lost activity during 24 hours incubation with hydrogen peroxide. Potassium ferricyanide did not decrease the activities of any of the proteins during the first two hours. Prothrombin and autoprothrombin C lost some activity during 24 hours incubation, but thrombin remained fully active. The compound 2,6-dichlorophenolindophenol destroyed the activity of all three in 24 hours. It was most destructive of prothrombin and autoprothrombin C. Within two hours approximately 30% of the prothrombin was gone. Activity of all three molecules was greatly reduced during 24 hours incubation.

Mercaptide forming reagents. Heavy metals, particularly mercury, arsenicals, and selenites, combine with single sulfhydryl groups under mild conditions. The mercury containing compounds which were used in this study included: p-chloromercuribenzoate, merthiolate, phenylmercuric nitrate, and phenylmercuric hydroxide.

These reagents exerted no inhibitory effect during the first two hours incubation with prothrombin, thrombin or autoprothrombin C. After 24 hours incubation, the only inhibitory effects noted were a decrease of 45% and 25% of prothrombin activity by phenylmercuric hydroxide and phenylmercuric nitrate, respectively.

The arsenic containing compounds employed were both pentavalent arsenicals: mapharside, and p-arsanilic acid. Mapharside had no effect upon the proteins tested. The p-arsanilic acid had the most inhibitory effect against autoprothrombin C, which lost 25% of its activity within the first two hours and 60% in 24 hours. Thrombin decreased in activity approximateiy 25% in two hours and nearly 50% in 24 hours. Prothrombin retained its activity during the 24 hours incubation with p-arsanilic acid.

Alkylating agents. The only reagent of this groups we studied was iodoacetic acid. In the presence of a 0.01 M concentration, the proteins were unaffected, with the exception of prothrombin, which lost 28% of its activity during a 24-hour incubation period. However, when the proteins were incubated in a 0.05 M solution of neutral iodoacetic acid, thrombin activity decreased about 12% within the first two hours, then remained constant during a 24-hour period of incubation. Although prothrombin lost no activity during the first two hours, a 70% decrease occurred during the 24-hour period. Autoprothrombin C activity decreased 15% during the first two hours and about 55% during the 24-hour incubation period.

Amino group reagents. Representing this group of reagents were: formaldehyde solution, acetylmercaptosuccinic anhydride. Prothrombin activity proved to be quickly decreased in the presence of formaldehyde. Thrombin activity dropped nearly 25% in the first two hours and did not decrease further during the next 22 hours incubation. Autoprothrombin C was resistant to inhibition by formaldehyde during the first two hour incubation period and lost only about 15% during 24 hours incubation.

Prothrombin and thrombin activities were lost during two hours incubation with acetylmercaptosuccinic anhydride. Autoprothrombin C activity was unaffected following two hours incubation with this reagent, but nearly all activity disappeared following 24 hours incubation.

Carbonyl group reagents. Three reagents were employed to study the possibility of carbonyl groups being essential groups in prothrombin, thrombin and autoprothrombin C: phenylhydrazine hydrochloride, hydroxylamine sulfate, and sodium bisulfite. Activity of all three proteins decreased over 75% during 24 hours incubation with phenylhydrazine. Autoprothrombin C activity decreased approximately 45% within the first two hours, prothrombin approximately 30% and thrombin approximately 20%. Thrombin activity was stable during a 24 hour incubation period with hydroxylamine. Autoprothrombin C activity fell approximately 40% during two hours incubation and 80 to 85% during 24 hours. Prothrombin activity diminished 15 to 20% during two hours incubation and between 70 and 80% after a 24-hour incubation period with hydroxylamine.

The prothrombin and autoprothrombin C activity was rapidly lost in contact with sodium bisulfite. Thrombin was somewhat affected by sodium bisulfite in two hours; however 24 hours was required to show a marked inhibitory effect upon the ability of thrombin to clot fibrinogen.

Miscellaneous reagents. Four reagents were tested which do not nicely fall into any of the above categories: phenol, lithium bromide, guanidine hydrochloride, and potassium ferrocyanide.

Phenol, in a 1% solution, did not destroy the activity of prothrombin, thrombin or autoprothrombin C during a 24-hour incubation period. Lithium bromide and guanidine hydrochloride, in the low concentrations used, did not affect the activity. Potassium ferrocyanide was not very effective in decreasing the activity of prothrombin or autoprothrombin C. Activity was unaffected after two hours but, after 24 hours, up to 25% of the activity was lost. Nearly 50% of the thrombin activity disappeared after two hours incubation with this agent and nearly 75% after 24 hours.

Discussion

Disulfide Group

Reducing agents act on unmodified proteins to reduce disulfide bonds to sulfhydryl groups. Those reducing reagents containing sulfhydryl groups are very specific in their action on disulfide bonds: on the other hand, they activate enzymes requiring sulfhydryl groups (18). Sulfites and bisulfites add to disulfide bonds to give a sulfhydryl and thiosulfite (6).

In this series of investigations reducing the disulfide bond did not decrease thrombin activity in the regular concentrations we were using. By increasing the concentration to 0.2 M inactivation was observed. At 0.05 M the results were variable, and there was even spontaneous recovery of activity subsequent to inactivation. Both prothrombin and autoprothrombin C activity rapidly decreased following reduction of the disulfide bond, which indicates disulfide bondings are essential for their activity. This agrees with the statements of Carter and Warner (4, 5) concerning prothrombin and thrombin, with Koppel, *et al.* (8, 9) concerning prothrombin and Nanninga's (15) investigation of thrombin, with the exception of the observation that thrombin can be inactivated if the concentration of reducing agent is high enough.

Sulfhydryl Groups

Sulfhydryl groups oxidize readily to disulfides. However, the only reagents, which can be considered to specifically oxidize sulfhydryls, are disulfide reagents (3). The disulfide oxidizing reagents used in this investigation did not

decrease the activity of prothrombin, thrombin or autoprothrombin C. On the other hand, strong oxidizing agents markedly decreased activity of all three proteins, and since a low degree of inactivation was seen in all three following incubation with mild oxidizing agents, it must be assumed that this effect was due to oxidation of groups other than sulfhydryl. This does not exclude the essential position of sulfhydryl groups, however, since these groups must be freely reacting and be located close together in order for oxidation to disulfides to occur.

Mercaptide forming reagents are able not only to react with free sulfhydryl groups but are also able to react with sluggish ones. The heavy metals, mercury, arsenic and selenite combine with single sulfhydryl groups under mild conditions. Among this type of reagent, p-chloromercuribenzoate is one of the most widely used and probably most specific. It is able to combine with a single sulfhydryl group at low concentrations of reagent.

The trivalent arsenicals have a striking affinity for sulfhydryl groups. Mapharside has been used clinically in the past for treatment of trypanosomal and spirochetal infections. *In vivo* this pentavalent arsenical is converted to a trivalent form and then exerted its effect by blocking essential sulfhydryl containing enzymes (16); therefore, one would except that mapharside would block sulfhydryl groups only when used *in vivo*.

In the presence of sulfhydryl groups iodoacetic acid forms a thioether. At one time iodoacetic acid was thought to be specific for proteins which contained sulfhydryl groups. It is now realized that, during a longer incubation period, iodoacetate reacts with other groups, including amino, phenol and indole (14). During this investigation, the clotting proteins decreased in activity only in high concentrations and following long incubation, so most likely some group other than sulfhydryl was involved.

Considering the results of the three methods used here to study the essential position of sulfhydryl groups – that is, specific oxidizing agents which contain disulfide linkages, the mercaptide forming reagents, and rapid inactivation with iodoacetate – we have found that sulfhydryl groups are not essential for the clotting activities of prothrombin, thrombin, or autoprothrombin C.

Amino Group

Acetic anhydride is a valuable reagent for studying the essential nature of amino groups without causing denaturization of the protein, if the protein is kept cold and subjected to a short exposure period. More active or prolonged exposure will probably result in other than amino groups being involved. A method for acetylation of prothrombin and thrombin has been reported which does not denature either molecule but certainly changes their properties (11, 12).

Prothrombin, after being acetylated, is no longer able to give thrombin, which clots fibrinogen, upon activation with thromboplastin or concentrated sodium citrate. The prothrombin molecule, in contact with thromboplastin, however can be converted to acetylated thrombin. Acetylated thrombin no longer has the ability to clot fibrinogen but retains its ability to split synthetic substrates such as p-toluene sulfonyl-l-arginine methyl ester – that is, it has retained its esterolytic capacity. Previous publications of Seegers and Landaburu (23) have pointed to the fact that esterolytic activity of thrombin is not always coexisting with clotting activity. Normally thrombin has both esterolytic and clotting activity.

The fact that acetylated prothrombin is activated to give acetylated thrombin indicates that the amino groups acetylated in prothrombin are probably those which are acetylated in thrombin. Acetylated thrombin which is obtained from activation of acetylated prothrombin has the same characteristics as acetylated thrombin which has been obtained by acetylating isolated thrombin.

Acetylmercaptosuccinic anhydride has been reported to have a strong preference for amino groups (10). Prothrombin and thrombin activities were completely lost following two hours incubation with acetylmercaptosuccinic anhydride. Autoprothrombin C activity was unaffected following two hours incubation with acetylmercaptosuccinic anhydride, but nearly all of its activity was lost following 24 hours incubation with this reagent.

Formaldehyde in neutral solution reacts with free amino groups in an equilibrium type reaction. The reaction of formaldehyde with proteins is complicated and, although it cannot be considered a specific reagent, it is of value when considered in conjunction with other reagents. Prothrombin activity was markedly decreased by formaldehyde, thrombin activity was decreased slightly and autoprothrombin C activity was not affected until after a long incubation period.

Markwardt (13) found that as thrombin activity disappeared in formaldehyde hirudin binding capacity was diminished and, with the return of thrombin activity, hirudin binding capacity was restored. He also found that hirudin did not combine with acetylated thrombin. This was interpreted to indicate that an amino group of thrombin must be available to combine with hirudin. In another study we found that hirudin does not combine with autoprothrombin C, and here we are reporting that autoprothrombin C activity is not lost when incubated with reagents which bind amino groups.

Nitrous acid reacts with amino groups of proteins, but it also reacts with other groups including sulfhydryl and phenol. Although it cannot be considered a specific reagent, it is felt that, if it inhibits rapidly, the reaction is with amino groups and, if slowly, it is with the phenol groups. Seegers reported in 1940 (19)

that the thrombin and prothrombin activity was rapidly destroyed by dilute solution of nitrous acid at pH 5.5, which indicates that their activities were dependent upon the integrity of alpha amino groups.

Compiling the results of this present report, and those previously published, free amino groups are required for normal prothrombin and thrombin activity, but autoprothrombin C does not appear to require amino groups for its activity.

Carbonyl Group

Hydrazine and hydroxylamine are known to react with aldehyde and ketone groups. Prothrombin and autoprothrombin C activities were lost following incubation with both phenylhydrazine and hydroxylamine. The results with thrombin were variable in that it retained full activity following incubation with hydroxylamine, but decreased somewhat with phenylhydrazine.

In addition to reducing disulfide bonds, sodium bisulfite in a saturated aqueous solution will react to form addition compounds with aldehydes and methyl ketones (17). Although the sodium bisulfite incubated with prothrombin, thrombin and autoprothrombin C was only 0.05 M, the decreased activity noted could possibly have been a result of some combination with aldehyde or methyl ketone groups. Prothrombin and autoprothrombin C activities had the greatest decrease and thrombin activity was somewhat decreased.

Olcott and Fraenkel-Conrat (18) state that no protein group is known to react with hydroxylamine or hydrazines in the concentrations used and they draw the conclusion that few enzymes which are markedly inhibited by these low concentrations should be ascribed to the presence of prosthetic groups or coenzymes containing ketone or aldehyde groups. On the other hand, the inhibiting effect has not been adequately explained in all cases. The results found here indicate that prothrombin and autoprothrombin C activity is decreased by inhibiting aldehyde or ketone groups, and loss of thrombin activity is inconclusive.

Miscellaneous Reagents

Phenol solution did not decrease the activity of the clotting factors investigated. This does not give any information about the active sites on the molecules, but allows the use of phenol as a preservative agent in the prevention of bacterial contamination if such is desired.

Progressive inhibition of thrombin activity by potassium ferrocyanide is in agreement with the report by Nanninga (15).

Comparative Abilities of Various Reagent Groups to Inhibit Clotting factors

Table 2 contains a summary of the effect of various reagent groups on activities of five purified clotting factors. Included in the table are the results of

Table 2. Comparative Abilities of Various Reagent Groups to Inhibit Clotting Factors

	Prothrombin	Thrombin	Autoprothrombin C	Ac-globulin (factor V)	Autoprothrombin I (factor VII)	Platelet Cofactor I (factor VIII)
Reducing agents	+					
Sulfhydryl blocking agents						
Amino group	-	-	-	-	?	
Oxidizing agents						
a) Nonspecific	-	-	-	-		
b) Disulfide	+	+	+	-		
Carbonyl group	+	±	+	±		

+ Indicates that reagents used caused inhibition of the protein activity.
- Indicates that reagents used did not cause inhibition of protein activity.
? Indicates that the reagents were not tested with clotting factor.
± Results inconclusive. In the case of Ac-globulin hydroxylamine tended to stabilize.
1) Reference (1).
2) Reference (22).
3) Reference (2).

prothrombin, thrombin and autoprothrombin C reported in this publication; Ac-globulin (factor V) published by Aoki, *et al.* (1); autoprothrombin I published by Seegers and Kagami (22); and platelet cofactor I (factor VIII) published by Baker and Seegers (2).

In the table a plus indicates that the representative reagents of the group caused inhibition of the activity of the clotting factor. A plus also indicates that the group is essential for the activity of that clotting factor. From the table it can be noted that disulfide bonds which are readily reduced are essential for activities of prothrombin, autoprothombin C and autoprothrombin I (factor VII). Sulfhydryl groups are essential only for the activity of Ac-globulin. Prothrombin, thrombin, and platelet cofactor I (factor VIII) contain essential amino groups; autoprothrombin C does not; and it has not been determined for Ac-globulin or autoprothrombin I. A carbonyl group is essential for activities of prothrombin, autoprothrombin C, autoprothrombin I, and platelet cofactor I. All of the clotting factors are readily inactivated by oxidizing agents, but not by those oxidizing agents containing disulfide groups, which means that groups other than sulfhydryl were oxidized. Ac-globulin (factor V) is the exception. Due to its essential sulfhydryl group Ac-globulin readily loses activity by oxidizing agents containing disulfide groups.

Summary

Numerous chemical enzyme inhibitors were employed to determine the active polar groups in the prothrombin, thrombin and autoprothrombin C molecules. Prothrombin and autoprothrombin C activities were decreased by the reduction of disulfide bonds, but thrombin activity was affected only at higher concentrations of reducing agent. Amino groups were not essential for the activity of autoprothrombin C; however, the blocking of such groups decreased the activities of prothrombin and thrombin. The blocking of carbonyl groups decreased the activity of both prothrombin and autoprothrombin C, but thrombin was not inactivated by hydroxylamine. The activities of none of the three were decreased by sulfhydryl blocking agents. Nonspecific oxidizing agents reduced the activities of all three molecules; however, oxidizing agents which specifically oxidize sulfhydryl groups, were incapable of decreasing the activities of prothrombin, thrombin or autoprothrombin C. The sulfhydryl groups are not essential for the activities of prothrombin, thrombin or autoprothrombin C.

Résumé

On a utilisé de nombreux inhibiteurs d'enzyme afin de déterminer les groupes polaires actifs des molécules de prothrombine, thrombine et autoprothrombine C. L'activité de la prothrombine et celle de l'autoprothrombine C sont diminuées par la réduction des liaisons disulfures, l'activité de la thrombine n'est affectée qu'aux concentrations plus élevées de l'agent réducteur. Les groupes aminés ne sont pas essentiels pour l'activité de l'autoprothrombine C, par contre, le blocage de ces groupes diminue l'activité de la prothrombine et de la thrombine. Le blocage des groupes carbonyles fait diminuer l'activité de la prothrombine et de l'autoprothrombine C, mais la thrombine n'est pas inactivée par l'hydroxylamine. L'activité d'aucune des trois molécules n'est bloquée par les réactifs du groupe sulphydryle. Les agents oxydants non spécifiques réduisent l'activité des trois molécules, par contre les oxydants spécifiques des groupes sulphydryles sont incapables d'altérer l'activité de la prothrombine, de la thrombine et de l'autoprothrombine C. Les groupes sulphydryles ne sont pas essentiels pour l'activité de la prothrombine, de la thrombine et de l'autoprothrombine C.

Zusammenfassung

Zahlreiche chemische Fermenthemmstoffe wurden angewendet, um die aktiven polaren Gruppen in den Molekülen von Prothrombin, Thrombin und Autoprothrombin C zu bestimmen. Die Aktivität von Prothrombin und Autoprothrombin C wurde durch Reduktion der Disulfid-Bindungen vermindert, die

Aktivität von Thrombin wurde aber nur von wesentlich höheren Konzentrationen des reduzierenden Agens beeinflußt. Aminogruppen waren nicht von Wichtigkeit für die Aktivität von Autoprothrombin C; Blockierung solcher Gruppen hingegen verminderte die Aktivität von Prothrombin und Thrombin. Die Blockierung von Carbonylgruppen verminderte die Aktivität von Prothrombin und Autoprothrombin C, Thrombin hingegen wurde von Hydroxylamin nicht inaktiviert. Die Aktivität keiner der drei untersuchten Substanzen wurde durch sulfhydrylblockierende Agenzien vermindert. Unspezifisch oxydierende Agenzien reduzierten die Aktivität aller drei Moleküle; oxydierende Agenzien hingegen, welche in spezifischer Weise Sulfhydrylgruppen oxydieren, verminderten die Aktivität von Prothrombin, Thrombin oder Autoprothrombin C nicht. Sulfhydrylgruppen sind also nicht von Wichtigkeit für die Aktivität von Prothrombin, Thrombin und Autoprothrombin C.

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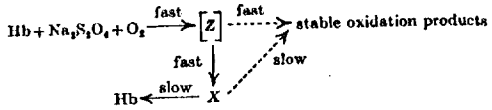
Side-reactions in the Deoxygenation of Dilute Haemoglobin Solutions by Dithionite. By K. DALZIEL and J. R. P. O'BRIEN. (*Department of Biochemistry, Radcliffe Infirmary, Oxford*)

Legge & Roughton (1950) found that an apparent slow terminal stage sometimes occurred in the deoxygenation of sheep haemoglobin solutions by dithionite at neutral pH, and obtained evidence for the formation of abnormal pigments by the action of oxidation products of dithionite, which could invalidate their measurements of the extent of deoxygenation with a Millikan colorimeter employing light filters. The specific cause was not established, but a precursor of choleglobin was suggested; a few experiments in which the composition of the reaction mixture was obtained from the light absorption at 578 mμ. showed no slow phase. In the course of studies of the deoxygenation by dithionite at pH 8·5 of human haemoglobin from normal and diseased persons, with a modified constant flow apparatus (Hartridge & Roughton, 1923) in which the course of the reaction is followed with a Beckman photoelectric spectrophotometer, we found no slow phase in forty kinetic experiments at 430 mμ. and in single experiments at 415, 465 and 560 mμ. Investigations of incidental observations made during this work form the subject of this communication: the results support the conclusions of Legge & Roughton, and throw some further light on the side-reactions involved.

Spectroscopic evidence has been obtained for the initiation of three reactions by the action of oxygen

on dilute solutions of human haemoglobin in dithionite. The net effect is decreased absorption at both haemoglobin maxima, and increased absorption at the 480 mμ. minimum and at 610–640 mμ., and is attributed to the formation of choleglobin and probably other oxidation products. These spectral changes are more pronounced immediately after the reaction with oxygen, but are partially reversed during the subsequent 5 min. Similar slow changes in spectral absorption follow the reduction of oxyhaemoglobin (and methaemoglobin) solutions by dithionite. These observations are attributed to the rapid formation of an unstable compound having isosbestic points with haemoglobin at about 417, 448, 535 and 579 mμ. (pH 8·5) and its subsequent rather slow reversion to haemoglobin. Parallel variation in the extent of the permanent and the transient spectral changes with pH, dithionite concentration and pigment concentration suggests either simultaneous side-reactions or a common intermediate:



Kinetically and spectroscopically, similar reactions follow the addition of small concentrations of hydrogen peroxide to dithionite solutions of haemoglobin, and a transient compound having isosbestic points with haemoglobin in close agreement with those of *X*, and an absorption maximum at 416–418 mμ. (pH 8·3) has been demonstrated. A similar absorption maximum has been obtained by the action of peroxide on dilute catalase-free oxyhaemoglobin solutions.

One of us (K.D.) wishes to acknowledge the receipt of a grant from the Haematology Research Fund.

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Chemical Prevention of Black Spots (Melanosis) In Frozen and Ice-stored Crustaceans by Rafael Establier

In a previous study, (R. Establier 1965) we carried out experiments using potassium metabisulphite in the preservation of ice-stored crustaceans. These experiments were done by powdering this compound in amounts of 1,000 and 1,500 grams per box of crustaceans weighing 35-40 kilos. Under these conditions it was proved that we could preserve crustaceans for a period of 3 to 15 days. After the indicated experiments, others were conducted by ship-owners of Cadiz obtaining excellent results in almost all the cases. Following which 45 boxes of gambas (type of crustacean) that were acquired by one of Cadez exporters, were unshipped in perfect condition by the ship "Olaveaga".

When these experiments were finished, by the request of fishing ship-owners of Cadiz and Huelva, it was agreed to continue this work on the preservation of crustaceans with the objective of developing methods for prevention of melanosis in frozen crustaceans and to increase the preservation time of ice-stored crustaceans. These experiments were supported by the Dirección General of Sanidad Veterinaria with their Jefaturas Provinciales of Cadiz and Huelva, the Asociaciones of Armadores of Buques of Pesca of Cadiz and Huelva and the Intituto of Investigaciones Pesqueras (Laboratorio of Cadiz) which carried out the technical directions of the experiments.

These experiments began on February 1966, with several experiments of ice preservation and freezing. We had to reject many of these experiments because or irregularities in the preparation which arrived in bad condition (generally without ice). In addition, a large number of projected experiments could not be carried through because samples were lost, generally in the ice-storage trawlers. In the experiments with frozen crustaceans we have used several dissolved products and in the ones done with ice we tried solutions and powders of solid products.

Experimental Section

Material and Methods used

All the chemicals used were commercialy obtained; potassium metabisulphite, sodium hydrosulphite were from the company Derivados del Azufre S.A. The sodium metabisulphite, ascorbic acid, sorbic acid, benzoic acid, sodium benzoate and citric acid, were also commercial products but we ignore their source.

The experimental samples were prepared in two ways, on board the trawlers. One was by powdering the chemicals in the way described previously (R. Establier 1965) and the other by preparing solutions and immersing the crustaceans for a definite period.

For the preparation of these last experiments, the foremen of the trawlers were given a plastic bathtub with a capacity of 25 liters which had specific volume level (10 to 15 liters) marked. In addition, they were given numbered bags containing the amount of the chemicals to prepare the desired volume and concentration. Once the solutions were prepared, 5 kilos of crustaceans were immersed and kept for the required period. After the crustaceans were treated with the solutions, they were put in a box with crushed ice or were frozen depending on the type of trawlers in which the experiments were carried through. In the ice-storage experiments like the ones by freezing, the experimental boxes were separated from the rest of the shipment that had been treated with boric acid and sodaic hyposulphite.

When these trawlers arrived at the harbour the samples were qualified macroscopically by a jury which was made up of representatives of the ship-owners, shell-fish exporters, a provincial Inspector of Veterinarian Health and a member Cadiz Laboratory of the Institute of Fishing Investigation, using the following system for evaluation: Very Good (VG), Good (G), Regular (R), Bad (B) and Very Bad (VB). Following, an analysis was conducted to determine the amount of SO_2 in the tissues of the crustaceans. The contents of freezing experiments samples were determined in volatile basis.

The determinations of volatile basis and SO_2 residual was by the methods of STANSBY and COL (1944) and MORNIER-WILLIAMS (1927) respectively, as described in a previous work (R. ESTABLIER 1965).

In the freezing experiments the samples analysed for SO_2 residual were then defrosted and put in crushed ice for 48 hours, following which the crustaceans were again examined macroscopically to see if they had been affected by melanosis during this period.

Analysis of Results

Table I summarizes 28 experiments carried through on board the freezer trawlers and Table II shows the results of the analysis of SO_2 residual and of the macroscopic examinations of the samples on arrival at the harbour following 48 hours in ice.

The chemicals used in prevention of melanosis of frozen crustaceans were: sodium hydrosulphite, sodium hyposulphite, potassium and sodium metabisulphite, citric

acid and the combination of citric-ascorbic acids. Following is a brief summary of the experiments done with these compounds:

The experiments with sodium hydrosulphite were 8 (experiments number 15, 20, 21, 22, 25, 26 and 28 from Table I) on board trawlers "Ubaldino Queiroz", "Jacinto Verdaguer", "Consuelo Veiga" and "Salvedina". In these experiments we have used 2.5, 3.5, 4, 4.5, 5, 6 and 7% solutions of sodium hydrosulphite with the same period of immersion (5 minutes). The periods of preservation of experiments with solutions of sodium hydrosulphite range between 39 and 93 days, showing that in all experiments they had excellent results (Table II) no symptons of melanosis was seen in any case, on harbour arrival or after 48 hours in ice, following defrosting.

The contents in SO_2 residual of these experiments have ranged between 5.98 and 18.10 mg SO_2/100 gr. tissue. In these residual contents we can appreciate some irregularities as in experiments 27 and 28 where the results are much too high compared to other experiments. These irregularities are probably due to keeping the samples immersed in the solutions for a longer period than stated in the instructions.

welve experiments with potassium metabisulphite were conducted on board the freezer trawlers in Cadiz "Salmedina", "Consuelo Veiga", "Ubaldino Queiroz" and "Esmeraldo Domínguez" with preservation periods ranging between 41 and 93 days, using 2 to 5% solutions and bathing periods of 2 to 7 minutes.

It has been proven that for the treatment to be effective a minimum concentration of 3% and a maximum of 4 to 5% is required with bathing periods of 3 to 5 minutes. In the 12 experiments conducted,only one showed melanosis in the crustaceans (experiment 1, Table II) in which a 2% solution was used, in the other experiments crustaceans were very well preserved on harbour arrival and after 48 hours in ice, following defrosting.

We should remark that experiments summarized under numbers 10 and 11 belong to shipments of around 10 tons of "Chorizo blanco" (Aristeus antennatus) and 1.5 tons of gambas (Parapenaeus longirostris Lucas) respectively. The "Chorizos blancos" like the gambas in these shipments arrived in perfect conditions and were normally sold.

Experiments 17, 18 and 19 were samples from a shipment of about 22 tons of crustaceans that were prepared with potassium metabisulphite by the ship-owner of the trawler "Esmeraldo Domínguez". These samples summarized in Table I, were supplied by the foreman of the ship and although the result of the analysis

of SO_2 residual was not as normal, they did not show symptoms of melanosis, even after 48 hours in ice. After the examination of samples that they gave us, it was seen that in the shipment of gambas, about 10 tons, some boxes were affected by melanosis, because they were not treated correctly on board. The rest of the shipment of crustaceans "Chorizos rojos" (Plesiopenaeus edwardsiansJ.) and "blancos" and "quisquilla" (Leander Sp) were normally sold.

In the experiments 8, 14 and 24 we used 3.5, 4, 5% solutions of sodium metabisulphite (see Table II) obtaining excellent results in the 3 experiments, with preservation periods of 39 to 93 days.

With sodium hyposulphite two experiments were conducted (4 and 9 from Table I) using 3 and 5% solutions. In experiment number 3, that was done with "Chorizo rojo", no symptoms of melanosis was seen. Number 9 was done with gambas after 68 days of preservation, the frozen crustaceans did not showed black spots after 48 hours in ice, it was estimated that 80% of gambas had black heads caused by melanosis.

The experiments done with citric acid (experiments 5 and 16) had the same results as the experiments with sodium hyposulphite, that is, the experiment done with "Chorizos rojos" was not affected by melanosis while the one with gambas, following 3 hours in ice, 70% of the crustaceans were black.

With the combination of citric-ascorbic acid only one experiment was conducted (number 3) with "Chorizos rojos" that gave a negative result with 20% melanosis in the crustaceans treated with these compounds.

Besides the experiments in freezer trawlers named in Table I, 8 experiments were done in the trawler "Cesar Cuiñas" of Huelva with solutions of potassium metabisulphite, sodium sulphite, sodium hyposulphite, citric ascorbic and glucose acids. In all these experiments gambas were affected by melanosis, including three experiments done with 2.5, 4 and 7% solutions of potassium metabisulphite with periods of immersion of 2 minutes. The results of the analysis of SO_2 residual were very low and all the samples had strong smell of petroleum, they have not been included with the conclusions since there were definitely some irregularities made in its preparation.

B. Ice-stored Crustaceans

In the experiments done on board ice-storage trawlers treatments were by immersion of crustaceans in solutions and powder of the chemicals.

Potassim metabisulphite and sodium hydrosulphite were used in the experiments of immersing the crustaceans in solutions. Table III shows results of the n analysis of SO_2 residual and contents in volatile basis, together, with estimations obtained in the macroscopic examinations of the crustaceans on arrival at the harbour.

Using 6.5 and 10% solutions of potassium metabisulphite, three experiments were done (experiments 1, 2 and 3, Table III). Experiments 1 and 2 were done with gambas, 6.5 and 10% solutions with a bathing periods of 2 to 3 minutes. Both experiments after 9 days of preservation on board, arrived in good condition, showing only (experiment number 1) 0.5% of black gambas because they were not covered properly with the solution. Experiment number 3 was done by immersing the sample of gambas in 10% solution of potassium metabisulphite for 5 minutes. After 17-18 days of preservation on board 5% of the gambas were black and the crustaceans were soft. We should remark that the sample had good smell and normal content in volatile basis, which shows that decomposition had not yet began after 17-18 days. This is because during the trip the sample was kept in a cool enough imperative as the ship in which the experiment was done "Apostol San Andrés" has climatized storage area. This was also proven with other samples of quisquillas (type of crustaceans) prepared by powder of potassium metabisulphite on the ship (experiment 21, Table IV) which had no melanosis after 17-18 days board and were qualified Very Good.

In table III are summarized 6 experiments done by immersing the crustaceans in solutions of sodium hydrosulphite and followed afterwards in ice (experiments 4 and 9). In these experiments they used 8, 10, 12 and 15% solutions of sodium hydrosulphite with treatment period s of 5 to 10 minutes.

In these experiments we observed no symptoms of melanosis except for experiments 4 and 5 where they were a small proportion of black spots (2 and 5%) because of irregularities in bathing of crustaceans. However, it was observed a tendency of softening of organic tissues in the heads of the "Chorizos" with lost of the hepatopancreas and separation of the gambas' heads.

Experiments with powder of the chemicals over the crustaceans and preservation in ice boxes are detailed in Table IV along with the results of the SO_2 residual analysis and volatile basis together with macroscopic examinations. In these experiments we have used combinations of sodium hyposulphite, potassium metabisulphite, benzoic and sorbic acids, sodium benzoate and individually potassium metabisulphite and sodium metabisulphite.

In experiments 1, 2 and 3 combinations of sodium hyposulphite, benzoic and sorbic acids, sodium benzoate were used in proportions shown in table IV. The three periments after 14 days of preservation on board showed negative results ecause 90% of crustaceans were affected by melanosis.

On the trawler "Navarra", experiments 6 to 10 were done by using potassium

metabisulphite and combinations of this product with benzoic acid and sodium benzoate (see Table IV). The gambas belonging to these 5 experiments arrived in a state of decomposition even though there were samples with only 7 days of preservation on board, all these were not counted because of irregularities in its preparation or defects in the preservation on board (lack of ice in all the boxes). In previous experiences (Establier 1965) and in the same experiments done afterwards (Table IV experiments 11, 12, 13, 17, 18 19, 20, 21 and 22) it was proved that crustaceans treated with potassium metabisulphite were well preserved by longer periods.

Experiments 4 and 5 done in the trawler "María Cordero" were carried through with potassium metabisulphite, with preservation perdios on board of 16, 17 and 19 days. These experiments showed that the gambas were not affected by melanosis but showed symptoms of being decomposed with total lack of ice during the experiments.

Experiments 11, 12 and 13 were done on the trawler "Queipo de Llano", the first two with potassium metabisulphite and number 13 with sodium metabisulphite, with 16 days of preservation of experiments 11 and 14 days for 12 and 13. Experiment number 11 was done with "Chorizo blanco"with no symtoms of melanosis after 16 days, and qualified as regular as the crustaceans had suffered softening organic tissues. Experiments 12 and 13 were done with gambas, with no symptoms of melanosis after 14 days of preservation (12 on board and 2 in storage). We should remark that boxes of these experiments had lots of ice and before being examined they were preserved 2 days in the storage at 2-40C.

Experiments 17, 18 and 19 were done on board the ship "Jumar" using potassium metabisulphite with preservation periods on board of 14, 13 and 12 days accordingly. The macroscopic examination showed 1.10 and 10% of melanosis probably due to irregularities in the distribution of the product, and qualified as Good and Very Good. The boxes of these experiments had enough ice.

Experiments 20 and 22 were done on board the trawlers "Virgen de la Caridad" and "Verdel" with "Chorizo rojo" and using potassium metabisulphite. No melanosis showed after 15 and 14 days respectively. The other characteristics were qualified as Good and Very Good except for smell and texture of experiment 22 that was Regular due to some "Chorizos" had crushed heads (the ones at the bottom of the box).

Experiment number 21 was done in the trawler with climatized storage "Apostol San Andrés" with quisquilla and using potassium metabisulphite. After 17-18 days of preservation on board it did not show symptoms of melanosis and was

as qualified Very Good.

Summary and Conclusions

28 experiments were done on board freezer trawlers with solutions of different chemical products with the objective of preventing melanosis in frozen crustaceans. Of the chemicals experimented with, excellent results were obtained with potassium metabisulphite, sodium hydrosulphite and sodium metabisulphite.

Using 3 to 7% solutions of these compounds and immersing the crustaceans in these solutions for 2 to 5 minutes, melanosis is effectively prevented in frozen crustaceans and following defrosting can be kept 48 hours in ice without showing symptoms of melanosis (Tables I and II). This period of 48 hours seems more than enough to do the manipulations necessary in the commercial uses of frozen ~~xxxx~~ crustaceans. The contents of SO_2 residual of these experiments has range between 4.15 and 18.10 mg of SO_2/100 gr., percentages that are way below the limits allowed.

In ice-stored trawlers 31 experiments were carried through, 9 using solutions and bathing the crustaceans and 22 by powder chemicals directly.

In the 9 experiments on immersing the crustaceans in solutions before being put ice, we have only used potassium metabisulphite and sodium hydrosulphite. Of the results stated in table III, it shows that blackening of crustaceans is prevented by use of 6.5 and 15% solutions with immersing periods of 3 to 15 minutes. However, in periods longer than 9 days of preservation on board, a tendency for softening of organic tissue in the heads of the "chorizos" has been noted.

Of the powder chemicals used in 22 experiments in the ice-stored trawlers positive results were obtained with potassium and sodium metabisulphite. Some of the experiments done cannot be taken into account (ship "Caridad" and "Navarra" and "María Cordero" Table IV) since the experimental boxes on arrival to harbour were lacking ice, not showing however, symptoms of melanosis, even after 19 days of preservation on board. The crustaceans of these experiments were in well advanced state of decomposition including experiments with only 7 days of preservation (Table IV, experiment 10).

In the experiments on board the trawlers "Queipo de Llano" "Jumar" "V. de la Caridad" (stet from 22/11/66) and "Apóstol San Andrés" very good results were obtained after 14, 15 and 17 days of preservation on board and it is necessary to remark that all these experiments were carefully done, having the boxes enough ice during the trip.

ıerefore, we arrived to the conclucion that there is no problem in prevention of .nelanosis of frozen crustaceans with bathing in solutions of potassium or sodium metabisulphite or sodium hydrosulphite; resulting in perfect preservation without any symptoms of melanosis even after 48 hours in ice following defrosting. With respect to ice-stored crustaceans we arrived to the conclusion that for periods above 12, 14 days of preservation of crustaceans on board the trawlers it is necessary that the boxes have during the trip enough ice, being almost imperative that ships have climatized storage areas.

These was proved in the experiments done on board the trawler "Apóstol San Andrés" in which a box of quisquillas treated with potassium metabisulphite after 17-18 days on board did not show symptoms of melanosis and was qualified as Very Good.

Acknowledgement

We would like to show our appreciation for the cooperation on this work by the Gentlemen R. Garrido and R. Cabrerizo, provincial inspectors of Veterinarian Health of Cadiz and Huelva, to D.M. Veiga, Chairman of the Cooperative of ship-owners of Fishing Vessels of Altura and Huelva to D.J. Gestoso, foreman of the Cofradía of Pescadores of Cadiz and to the ship-owners, foremen of the 8 trawlers where the experiments were conducted.

ırther we thank Mr. A. Fernandez of Undustrias Gaditanas del Frio S.A. Mr. E. Quintana of Crustaceos & de Andalucía, and Mr. D.J. Sibon, ship-owner of Freezer trawlers for their help.

Table I

Experiments conducted on board Freezer Trawlers

Experiment No.	Trawler	Date of departure	Date of arrival	days of preservation	crustacean	chemical used	Treatment and bathing period

Table II

Results of analysis and macroscopical examinations conducted in the experiments carried out on freezer trawlers

		Macroscopical examination frozen crustaceans					Macroscopical Exam after 48 hrs. in ice				
Experiment No.	SO_2 residual mgr/100 tissue	Melanosis %	Color	Smell	Taste	Texture	Melanosis %	Color	smell	taste	texture

MB = VG
B = G
R = R
B = M
VB = MM

Table III

Experiments conducted by immersion of crustaceans in solutions of potassium metabisulphite and sodium hydrosulphite on board the trawlers by ice-sotrage

Experiment No.	Trawler	Date of departure	Date of arrival	Days of preservation on board	crustaceans	chemical used	Conc. of Sol. and bathing period

SO_2 residual mg/100 gr.	Volatile basis mg/100 gr	Macroscopical examination on arrival to harbour				
		Melanosis %	Color	Smell	Taste	Texture

Table IV

Experiments conducted by powder of chemicals in ice-storage trawlers

Experiment No.	Trawler	Date of departure	Date of arrival	Days of preservation on board	Chemical used	Dosification	Crustacean

SO_2 residual mg/100 gr	Volatile basis mg/100fix	Macroscopical examination on arrival to harbour				
		Melanosis %	Color	Smell	Taste	Texture

Inv. Pesq.	33 (1)	Págs. 55-68	Enero 1969

Prevención química del ennegrecimiento (Melanosis) de los crustáceos congelados y conservados en hielo*

por

RAFAEL ESTABLIER **

En un trabajo anterior (R. ESTABLIER, 1965) dábamos a conocer las experiencias que realizamos empleando metabisulfito potásico en la conservación de crustáceos almacenados con hielo. Estos ensayos se hicieron espolvoreando este compuesto en proporciones de 1000 a 1500 gramos por caja de crustáceos de 35-40 kilos. En estas condiciones se comprobó que se podían conservar los crustáceos por un período de tiempo comprendido entre los 3 y 15 días después de haber sido preparadas las cajas experimentales. Con posterioridad a las experiencias indicadas se hicieron algunas más por cuenta de los armadores de Cádiz, obteniendo en la casi totalidad de los casos excelentes resultados. Así el barco «Olaveaga» desembarcó 45 cajas de gambas en perfectas condiciones que fueron adquiridas por uno de los exportadores de Cádiz.

Finalizadas ya estas experiencias se acordó, a petición de los armadores de buques de pesca de Cádiz y Huelva, seguir realizándolas en la conservación de crustáceos pero encaminadas principalmente a poner a punto un tratamiento para la prevención de la melanosis en los crustáceos congelados y a tratar de encontrar nuevos tratamientos para aumentar el tiempo de conservación de los crustáceos almacenados en hielo. En estas experiencias colaboraron la Dirección General de Sanidad Veterinaria con sus Jefaturas Provinciales de Cádiz y Huelva, las Asociaciones de Armadores de Buques de Pesca de Cádiz y Huelva y el Instituto de In-

* Recibido para su publicación el 4-XI-67.
** Laboratorio del Inst. de Invest. Pesqueras. Puerto Pesquero. Cádiz.

vestigaciones Pesqueras (Laboratorio de Cádiz), que fue el que llevó la dirección técnica de las experiencias.

Estos ensayos se empezaron a realizar a partir de febrero de 1966, haciéndose numerosas pruebas tanto de conservación por hielo como por congelación. Muchas de estas experiencias tuvimos que desecharlas ya que por irregularidades cometidas en la preparación, ajenas a nuestra voluntad, llegaron en malas condiciones (generalmente falta de hielo). Por otro lado, un buen número de las experiencias proyectadas no se pudieron realizar por pérdida de las muestras u olvido, principalmente en los barcos de conservación por hielo.

En las experiencias realizadas con crustáceos congelados hemos empleado diversos productos siempre en disolución y en las hechas con hielo se experimentaron también soluciones y espolvoreo de los productos sólidos.

PARTE EXPERIMENTAL

Material y métodos empleados

Todos los productos empleados han sido de tipo comercial, así el metabisulfito potásico, hidrosulfito sódico e hiposulfito sódico procedían de la firma Derivados del Azufre, S. A. El metabisulfito sódico, ácido ascórbico, ácido sórbico, ácido benzoico, benzoato sódico y ácido cítrico también eran productos comerciales pero ignoramos su procedencia.

La preparación de las muestras experimentales se hizo a bordo de los pesqueros de dos formas. Una expolvoreando los productos de la manera descrita en un trabajo anterior (R. Establier, 1965) y la otra preparando soluciones de los productos e introduciendo en ellas los crustáceos durante un tiempo determinado.

Para la preparación de estos últimos ensayos se les entregó a los contramaestres o patrones de las embarcaciones un baño de plástico de unos 25 litros de capacidad que tenía marcado un nivel que correspondía a un volumen determinado (10 a 15 litros). Asimismo se les entregaba también bolsas numeradas conteniendo la cantidad del producto correspondiente para preparar el volumen marcado en el baño a la concentración deseada. Una vez preparadas las soluciones, se introducían dentro del baño porciones de 5 kilos de crustáceos y se mantenían dentro de él el tiempo indicado. Una vez tratados los crustáceos con las soluciones se pusieron éstos en una caja con hielo triturado o se congelaron según el tipo de barco en que se realizaran las experiencias. Tanto en los ensayos de conservación por hielo como en los hechos por congelación, una vez preparadas las cajas experimentales se colocaron separadas del resto del cargamento que había sido tratado con ácido bórico e hiposulfito sódico.

Una vez llegados a puerto los barcos que habían preparado las cajas experimentales, se calificaron organolépticamente las muestras por un jurado que en la casi totalidad de las experiencias estaba compuesto por un representante de los armadores, un exportador de mariscos, el inspector provincial de Sanidad Veterinaria y un miembro del Laboratorio de Cádiz del Instituto de Investigaciones Pesqueras, empleando el siguiente sistema de graduación: Muy Bueno (MB), Bueno (B), Regular (R), Malo (M) y Muy Malo (MM). Procediéndose posteriormente a efectuar los oportunos análisis para determinar el contenido en SO_2 retenido en los tejidos de los crustáceos. A las muestras correspondientes a los ensayos de conservación por hielo se les determinó también el contenido en bases volátiles.

Las determinaciones de bases volátiles y SO_2 residual se hicieron por los métodos de STANSBY y col. (1944) y MORNIER-WILLIANS (1927) respectivamente de la forma descrita en un trabajo anterior (R. ESTABLIER, 1965).

En las experiencias hechas por congelación, una vez tomadas las muestras para realizar los análisis de SO_2 residual, se descongelaron y se pusieron con hielo triturado durante 18 horas. Una vez pasado este tiempo, se volvieron a examinar organolépticamente los crustáceos al objeto de determinar si habían sido afectados de melanosis éstos durante este período.

ANÁLISIS DE LOS RESULTADOS

A. *Crustáceos congelados.*

En la tabla n.° I están resumidas las 28 experiencias realizadas a bordo de los barcos congeladores y en la tabla n.° II se dan los resultados de los análisis de SO_2 residual y de los exámenes organolépticos de las muestras a la llegada a puerto de las mismas y después de conservadas 48 horas en hielo.

Los productos empleados en la prevención de la melanosis de los crustáceos congelados han sido: hidrosulfito sódico, hiposulfito sódico, metabisulfito potásico y sódico, ácido cítrico y la mezcla de ácidos cítrico-ascórbico. A continuación se hace un breve resumen de los ensayos realizados con estos compuestos.

Los ensayos realizados con hidrosulfito sódico han sido 8 (ensayos n.° 15, 20, 21, 22, 25, 26, 27 y 28 de la tabla n.° I), efectuados en los barcos «Ubaldino Queiroz», «Jacinto Verdaguer» (2 mareas), «Consuelo Veiga» y «Salmedina». En estas experiencias se han utilizado soluciones de hidrosulfito sódico al 2,5, 3,5, 4, 4,5, 5, 6 y 7 %, habiéndose mantenido en todos los ensayos constante el tiempo de inmersión (5 minutos). Los

TABLA I

Ensayos realizados a bordo de barcos congeladores

Ensayo n.º	Barco	Fecha salida	Fecha llegada	Días de conserv.	Crustáceo	Producto empleado	Tratamiento y duración baño
1	Salmedina	1- 2-66	2- 4-66	89	Gambas	Metabisulf. Potásico	Sol. 2 % 3 minutos
2	»	1- 2-66	2- 4-66	88	Gambas	Metabisulf. Potásico	Sol. 4 % 3 »
3	C. Veiga	2- 1-66	6- 6-66	76	Chorizos	Ác. Cítrico.-Ác. Ascór.	Sol. 0.5 % 2 »
4	»	2- 1-66	6- 6-66	76	Chorizos	Hiposulfito Sódico	Sol. 3 % 2 »
5	»	2- 1-66	6- 6-66	76	Chorizos	Ácido Cítrico	Sol. 1.5 % 2 »
6	»	11- 6-66	23- 8-66	68	Gambas	Metabisulf. Potásico	Sol. 3 % 2 »
7	»	11- 6-66	23- 8-66	68	Gambas	Metabisulf. Potásico	Sol. 5 % 2 »
8	»	11- 6-66	23- 8-66	68	Gambas	Metabisulfito Sódico	Sol. 5 % 2 »
9	»	11- 6-66	23- 8-66	68	Gambas	Hiposulfito Sódico	Sol. 5 % 2 »
10	»	11- 6-66	23- 8-66	68	Choriz. bl.	Metabisulf. Potásico	Sol. 5 % 5-7 »
11	»	11- 6-66	23- 8-66	68	Gambas	Metabisulf. Potásico	Sol. 5 % 5-7 »
12	Ubaldino Queiroz	17- 7-66	1-10-66	93	Gambas	Metabisulf. Potásico	Sol. 2.5 % 3 »
13	» »	17- 7-66	1-10-66	93	Gambas	Metabisulf. Potásico	Sol. 4 % 3 »
14	» »	17- 7-66	1-10-66	93	Gambas	Metabisulfito Sódico	Sol. 3.5 % 3 »
15	» »	17 7-66	1-10-66	93	Gambas	Hidrosulfito Sódico	Sol. 2.5 % 3 »
16	» »	17- 7-66	1-10-66	93	Gambas	Ácido Cítrico	Sol. 1 % 3 »
17	Esmeraldo Doming.	25-10-66	12-12-66	41	Gambas	Metabisulf. Potásico	Sol. 5 % 5 »
18	» »	25-10-66	12-12-66	41	Choriz. roj.	Metabisulf. Potásico	Sol. 5 % 5 »
19	» »	25-10-66	12-12-66	41	Choriz. bl.	Metabisulf. Potásico	Sol. 5 % 5 »
20	Jacinto Verdaguer	8- 2-67	26- 3-67	39	Gambas	Hidrosulfito Sódico	Sol. 4 % 5 »
21	» »	8- 2-67	26- 3-67	39	Gambas	Hidrosulfito Sódico	Sol. 4.5 % 5 »
22	» »	8- 2-67	26- 3-67	39	Gambas	Hidrosulfito Sódico	Sol. 5 % 5 »
23	» »	8- 2-67	26- 3-67	39	Gambas	Metabisulf. Potásico	Sol. 5 % 5 »
24	Consuelo Veiga	9- 1-67	27- 3-67	65	Gambas	Metabisulfito Sódico	Sol. 4 % 5 »
25	» »	9- 1-67	27- 3-67	65	Gambas	Hidrosulfito Sódico	Sol. 4 % 5 »
26	Jacinto Verdaguer	29- 3-67	15- 5-67	42	Gambas	Hidrosulfito Sódico	Sol. 6 % 5 »
27	» »	29- 3-67	15- 5-67	42	Gambas	Hidrosulfito Sódico	Sol. 7 % 5 »
28	Salmedina	23- 2-67	1- 6-67	60	Gambas	Hidrosulfito Sódico	Sol. 3.5 % 5 »

TABLA II

Resultados de los análisis y exámenes organolépticos efectuados en las experiencias realizadas a bordo de los barcos congeladores

Ensayo n.º	SO_2 residual mgr. 100 Tejido	Examen Organoléptico Crustáceos Congelados					Examen Organoléptico después de 48 horas en hielo				
		Melanosis %	Color	Olor	Sabor	Textura	Melanosis %	Color	Olor	Sabor	Textura
1	2,5	0	MB	MB	MB	MB	30	B	MB	MB	MB
2	8,31	0	MB	MB	MB	MB	0	MB	MB	MB	MB
3		0	MB	MB	B	B	20	B	B	B	B
4		0	MB	MB	B	B	0	B	B	B	B
5		0	MB	MB	B	B	0	B	B	B	B
6	6,00	0	MB	MB	MB	MB	2	B	MB	MB	MB
7	8,16	0	MB	MB	MB	MB	0	MB	MB	MB	MB
8	8,20	0	MB	MB	MB	MB	0	MB	MB	MB	MB
9		0	MB	MB	MB	MB	80	M	B	MB	B
10	14,60	0	MB	MB	MB	MB	0	MB	MB	MB	MB
11	9,90	0	MB	MB	MB	MB	0	MB	MB	MB	MB
12	8,00	0	MB	MB	MB	MB	0	MB	MB	MB	MB
13	9,73	0	MB	MB	MB	MB	0	MB	MB	MB	MB
14	8,88	0	MB	MB	MB	MB	0	MB	MB	MB	MB
15		0	MB	MB	MB	MB	0	MB	MB	MB	MB
16		0	MB	MB	MB	MB	70	M	B	B	MB
17	5,65	0	MB	MB	MB	MB	0	MB	MB	MB	MB
18	4,15	0	MB	MB	MB	MB	0	MB	MB	MB	MB
19	6,80	0	MB	MB	MB	MB	0	MB	MB	MB	MB
20	6,90	0	MB	MB	MB	MB	0	MB	MB	MB	MB
21	6,61	0	MB	MB	MB	MB	0	MB	MB	MB	MB
22	10,23	0	MB	MB	MB	MB	0	MB	MB	MB	MB
23	8,01	0	MB	MB	MB	MB	0	MB	MB	MB	MB
24	6,32	0	MB	MB	MB	MB	0	MB	MB	MB	MB
25	5,98	0	MB	MB	MB	MB	0	MB	MB	MB	MB
26	8,90	0	MB	MB	MB	MB	0	MB	MB	MB	MB
27	18,10	0	MB	MB	MB	MB	0	MB	MB	MB	MB
28	13,05	0	MB	MB	MB	MB	0	MB	MB	MB	MB

tiempos de conservación de las experiencias realizadas con soluciones de hidrosulfito sódico han oscilado entre los 39 y 93 días, viéndose que todas las pruebas efectuadas han dado excelentes resultados (ver tabla n.º 11) ya que en ningún caso se han apreciado síntomas de melanosis ni a la llegada a puerto ni después de mantener los crustáceos, una vez descongelados, 48 horas en hielo. Los contenidos en SO_2 residual de estas experiencias han oscilado entre 5,98 y 18,10 mg SO_2/100 gr tejido. En dichos contenidos residuales se aprecian algunas irregularidades tal como las de los ensayos 27 y 28, cuyos análisis dan resultados excesivamente altos con relación a los restantes ensayos. Estas anomalías son probablemente debidas a mantener las muestras sumergidas en las soluciones un tiempo superior al indicado en las instrucciones dadas para la realización de los correspondientes ensayos.

Con metabisulfito potásico se controlaron 12 experiencias a bordo de los barcos congeladores con base en Cádiz, «Salmedina», «Consuelo Veiga», «Ubaldino Queirez» y «Esmeraldo Domínguez», con tiempos de conservación comprendidos entre los 41 y 93 días. Las soluciones empleadas de metabisulfito potásico han sido del 2 al 5 % y los tiempos de baño de 2 a 7 minutos. Se ha comprobado que son necesarias concentraciones mínimas del 3 % para que sean efectivos los tratamientos, siendo el óptimo emplear soluciones del 4 al 5 % y tiempos de baño de los crustáceos de 3 a 5 minutos. En las 12 experiencias realizadas, tan sólo se han apreciado crustáceos afectados de melanosis en una de ellas (ensayo n.º 1, tabla II), que se empleó solución al 2 %, en las restantes experiencias se han conservado los crustáceos muy bien tanto en las apreciaciones de la llegada a puerto como después de conservados, una vez descongeladas las muestras, 48 horas en hielo.

Es de hacer notar que los ensayos reseñados con los números 10 y 11 corresponden a partidas de unas 10 toneladas de «Chorizo blanco» (*Aristeus antennatus*, *R.*) y 1,5 toneladas de gambas (*Parapenaeus longirostris* Lucas) respectivamente. Tanto los «Chorizos blancos» como las gambas correspondientes a estas partidas llegaron, en su totalidad, en perfectas condiciones y fueron vendidos normalmente. Los ensayos 17, 18 y 19 corresponden a muestras representativas de un cargamento de unas 22 toneladas de crustáceos que fueron preparadas con metabisulfito potásico por iniciativa del armador del barco «Esmeraldo Domínguez». Dichas muestras, reseñadas en la tabla n.º I, nos fueron suministradas por el contramaestre de la embarcación y aunque el resultado de los análisis de SO_2 residual era ligeramente inferior a lo normal, no presentaron síntomas de melanosis ni aún después de 48 horas en hielo. Con posterioridad al examen de las muestras que nos entregaron se vio que en la partida de gambas, unas 10 toneladas, había algunas cajas afectadas de melanosis, debido a que no fueron tratadas correctamente a bordo. El resto de la partida de crustáceos «Chorizos rojos» [*Plesiopenaeus edward-*

sianus (J)] y «blancos» y quisquilla *(Leander Sp)* fueron vendidos normalmente.

En los ensayos 8, 14 y 24 se emplearon soluciones al 3,5, 4 y 5 % de metabisulfito sódico (ver tabla n.° II), obteniéndose en las tres experiencias realizadas excelentes resultados, con tiempos de conservación de 39 a 93 días.

Con hiposulfito sódico se realizaron dos ensayos (1 y 9 de la tabla n.° 1) empleando soluciones al 3 y al 5 %. En el ensayo n.° 3, que se realizó con «Chorizo rojo», no se apreciaron síntomas de melanosis. En el n.° 9 que se hizo con gambas, después de 68 días de conservación, no se apreciaron manchas en los crustáceos congelados, pero después de mantenerlos 48 horas en hielo se estimó un 80 % de gambas con la cabeza ennegrecida por efecto de la melanosis.

Con los ensayos efectuados utilizando soluciones de ácido cítrico (ensayos 5 y 16), ocurrió lo mismo que con las pruebas hechas con hiposulfito sódico, es decir, que el ensayo realizado con «Chorizos rojos» no fue afectado de melanosis mientras que en el realizado con gambas, después de mantener la muestra 48 horas en hielo, se le apreció un 70 % de crustáceos ennegrecidos.

Con la mezcla de ácido cítrico-ácido ascórbico se realizó un solo ensayo (el n.° 3) con «Chorizos rojos» que dio resultado negativo ya que se apreció un 20 % de melanosis en los crustáceos tratados con estos compuestos.

Aparte de los ensayos en buques congeladores especificados en la tabla n.° I se realizaron en el barco «César Cuiñas» de Huelva, 8 experiencias con soluciones de metasulfito potásico, sulfito sódico, hiposulfito sódico, ácidos cítrico y ascórbico y glucosa. En estos ensayos se apreció en todos gambas afectadas de melanosis, incluso en los tres ensayos hechos con soluciones de metabisulfito potásico a las concentraciones del 2,5, 4 y 7 % y tiempo de inmersión de 2 minutos. Como quiera que los resultados de los análisis de SO_2 residual eran muy bajos y todas las muestras presentaban fuerte olor a petróleo, no se han tenido en cuenta al sacar las conclusiones, ya que indudablemente se cometieron irregularidades en su preparación.

B. *Crustáceos conservados por hielo.*

En las experiencias realizadas a bordo de barcos de conservación por hielo se han ensayado tratamientos por inmersión de los crustáceos en soluciones de los productos empleados y por espolvoreo.

En los ensayos realizados sumergiendo los crustáceos en soluciones, se han empleado únicamente metabisulfito potásico e hidrosulfito sódico. En la tabla n.° III se dan las experiencias de este tipo que se han realizado así como los resultados de los análisis de SO_2 residual y contenido

TABLA

Experiencias realizadas por inmersión de los crustáceos en soluciones de Metabi

Ensayo n.º	Barco	Fecha salida	Fecha llegada	Días conserv. a bordo	Crustáceos	Producto empleado
1	Francisco Correa	4- 6-66	14-6-66	9	Gambas	Metabisulf. Potásic
2	Francisco Correa	4- 6-66	14-6-66	9	Gambas	Metabisulf. Potásic
3	Apóstol San Andrés	13-12-66	3-1-67	17-18	Gambas	Metabisulf. Potásic
4	A. Campelo	13- 2-67	3-3-67	16	Chorizos	Hidrosulfito Sódic
5	A. Campelo	13- 2-67	3-3-67	11	Gambas	Hidrosulfito Sódic
6	Noroeste	27- 2-67	15-3-67	14	Chorizos	Hidrosulfito Sódic
7	Verdel	23- 5-67	12-6-67	11	Gambas	Hidrosulfito Sódic
8	Verdel	23- 5-67	12-6-67	11	Chorizos	Hidrosulfito Sódic
9	Verdel	22- 6-67	19-7-67	15-16	Chorizos	Hidrosulfito Sódic

en bases volátiles, junto con las estimaciones obtenidas en los exámenes organolépticos de los crustáceos a la llegada a puerto de las cajas experimentales.

Empleando soluciones al 6,5 y 10 % de metabisulfito potásico se han realizado 3 experiencias (ensayos 1, 2 y 3 de la tabla n.º III). Los ensayos 1 y 2 se realizaron con gambas y soluciones al 6,5 y 10 % con una duración del baño de 2 a 3 minutos; ambos ensayos, después de 9 días de conservación a bordo, llegaron en buenas condiciones, apreciándose tan solo en el ensayo n.º 1 un 0,5 % de gambas negras debido, probablemente, a que no se cubrieron bien con la solución. El ensayo n.º 3 se hizo sumergiendo las gambas de la muestra en solución al 10 % de metabisulfito potásico durante 5 minutos. Después de 17-18 días de conservación a bordo se apreció un 5 % de gambas negras y que los crustáceos se encontraban algo blandos, viéndose también que la muestra se había preparado con gambas recién mudadas por lo que la apreciación de la textura no se podía hacer correctamente. Es de hacer notar que esta muestra presentaba buen olor y un contenido en bases volátiles normal, lo que indica que después de 17-18 días no había comenzado aún la descomposición. Esto es debido a que a la muestra no le faltó durante toda la travesía ni hielo ni el frío necesario ya que el barco donde se realizó la experiencia («Apóstol San Andrés») tiene las bodegas climatizadas. Este hecho se comprobó también en otras muestras de quisquillas, que se preparó expolvoreando metabisulfito potásico en la misma marea de este barco (ensayo 21; tabla n.º 4) que después de 17-18 días de permanencia a bordo no se le apreció melanosis y se calificaron de Muy Buenas las restantes apreciaciones organolépticas.

En la tabla n.º III están detallados los 6 ensayos que se han reali-

III

sulfito Potásico e Hidrosulfito Sódico a bordo de barcos de Conservación por hielo

Conc. de la Sol. y duración del baño	SO_2 residual mg 100 gr	Bases Volat. mg n 100 gr	Melanosis %	Examen Organoléptico a la llegada a puerto			
				Color	Olor	Sabor	Textura
Sol. 6,5 % 2-3 minutos	6,52	8,21	0,5	B	MB	MB	B
Sol. 10 % 2-3	7,20	5,76	0	B	MB	MB	B
Sol. 10 % 5	28,9	13,06	5	B	B	B	R
Sol. 8 % 5	4,95	18,6	2	B	B	B	R
Sol. 10 % 5	4,85	8,82	5	B	B	B	R
Sol. 10 % 5	7,35	24,08	0	B	B	B	R
Sol. 10 % 10	10,06	10,52	0	MB	MB	MB	B
Sol. 12 % 12	26,10	14,17	0	MB	MB	MB	B
Sol. 15 % 5	20,57		0	B	B	B	R

zado sumergiendo los crustáceos en soluciones de hidrosulfito sódico y colocados posteriormente en hielo (ensayos 1 al 9). En estas experiencias se han empleado soluciones de hidrosulfito sódico al 8, 10, 12 y 15 % con una duración de los tratamientos de 5 a 10 minutos. En estos ensayos se ha observado que se previene bastante bien la melanosis de los crustáceos ya que, exceptuando los ensayos 4 y 5 en las que se apreciaron pequeñas proporciones de ennegrecimiento (2 y 5 %) achacables a deficiencias en el baño de los crustáceos, en las restantes experiencias no se apreciaban síntomas de melanosis. No obstante se han observado en estos ensayos una tendencia al reblandecimiento de las cabezas de los «Chorizos», con pérdida de hepatopáncreas y a desprenderse con más facilidad las cabezas de las gambas.

Las experiencias realizadas por espolvoreo de los productos sobre los crustáceos y posterior conservación en cajas con hielo se encuentran especificadas en la tabla n.° IV, así como los resultados de los análisis de SO_2 residual y bases volátiles junto con las apreciaciones organolépticas. En estos ensayos hemos empleado mezclas de hiposulfito sódico y metabisulfito potásico con ácidos benzoico y sórbico, y benzoato sódico e individualmente metabisulfito potásico y metabisulfito sódico.

En los ensayos 1, 2 y 3 se emplearon mezclas de hiposulfito sódico y ácido benzoico y sórbico y benzoato sódico en las proporciones indicadas en la tabla n.° 1. Las tres experiencias después de 14 días de conservación a bordo dieron resultado negativo ya que se apreciaron en los tres ensayos un 90 % de crustáceos afectados de melanosis.

En el barco «Navarra» se realizaron los ensayos 6 al 10, empleando metabisulfito potásico y mezclas de este producto con ácido benzoico y benzoato sódico (ver tabla n.° IV). Las gambas correspondientes a estas

TABLA

Ensayos realizados por expolvoreo

Ensayo n.	Barco	Fecha salida	Fecha llegada	Días Conserv. a bordo	Producto empleado
1	Manolo Sibón	24- 4-66	14- 5-66	14	Hidrosulfito Sódico Benzoato Sódico
2	» »	24- 4-66	14- 5-66	14	Hidrosulfito Sódico Ácido benzoico
3	» »	24- 4-66	14- 5-66	14	Hidrosulf. Sód. Ácido Sórb.
4	V. Caridad	7- 6-66	27- 6-66	16	Metabisulfito Potásico
5	»	7- 6-66	27- 6-66	16	Metabisulfito Sódico
6	Navarra	7- 7-66	24- 7-66	13	Metabisulfito Potásico
7	»	7- 7-66	24- 7-66	13	Metabisulfito Potásico Benzoato Sódico
8	»	7- 7-66	24- 7-66	13	Metab. potásico + ác. benzoico + benzoato Sódico
9	»	7- 7-66	24- 7-66	10	Metabisulfito Potásico
10	»	7- 7-66	24- 7-66	7	Metabisulfito Potásico
11	Quelpo de Llano	1- 8-66	17- 8-66	16	Metabisulfito Potásico
12	» » »	1- 8-66	17- 8-66	14	Metabisulfito Potásico
13	» » »	1- 8-66	17- 8-66	14	Metabisulfito Sódico
14	María Cordero	1- 8-66	22- 8-66	19	Metabisulfito Potásico
15	» »	1- 8-66	22- 8-66	17	Metabisulfito Potásico
16	» »	1- 8-66	22- 8-66	17	Metabisulfito Sódico
17	Jumar	12-11-66	1-12-66	14	Metabisulfito Potásico
18	»	12-11-66	1-12-66	13	Metabisulfito Potásico
19	»	12-11-66	1-12-66	12	Metabisulfito Potásico
20	V. Caridad	22-11-66	9-12-66	15	Metabisulfito Potásico
21	Apóstol San Andrés	13-12-66	3- 1-67	17-18	Metabisulfito Potásico
22	Verdel	5- 1-67	23- 1-67	14	Metabisulfito Potásico

5 experiencias llegaron en avanzado estado de descomposición y como quiera que había muestras con sólo 7 días de conservación a bordo, se desecharon todas estas experiencias por irregularidades en la preparación o defectos en la conservación a bordo (falta casi total de hielo en todas las cajas) ya que en experiencias previas (ESTABLIER, 1965) y en los mismos ensayos que se realizaron posteriormente (tabla n.° IV, ensayos 11, 12, 13, 17, 18, 19, 20, 21 y 22) se había comprobado que los crustáceos tratados con metabisulfito potásico se conservaban bien por tiempos muy superiores.

Los ensayos 4 y 5 hechos en el barco «Virgen de la Caridad» y los 14, 15 y 16 efectuados en el barco «María Cordero» se realizaron con metabisulfito potásico, con tiempos de conservación a bordo de 16, 17 y 19 días. En estas experiencias se apreció que las gambas no habían sido afectadas de melanosis pero presentaban síntomas de estar descompuestas, siendo de notar también la carencia total de hielo en todos los ensayos.

I V

en barcos de conservación por hielo

Dosificación	Crustáceo	SO_2 residual mg/100 gr	Bases Volal. mg N/100 fix	Examen Organoléptico a la llegada a puerto				
				Melanosis %	Color	Olor	Sabor	Textura
170 gr+10 gr/5 kgr	Gambas	—		90	MM	R	R	R
170 gr+10 gr/5 kgr	Gambas	—		90	MM	R	R	R
170 gr+10 gr/5 kgr	Gambas	—		90	MM	R	R	R
225 gr/5 kgr	Gambas	50,85	40,32	0	B	R	M	R
225 gr/5 kgr	Gambas	53,02	16,75	0	B	R	M	R
500 gr/10 kg	Gambas	52,87	Descompp.	10	R	MM	R	M
450 gr+10 gr/10 kg	Gambas	49,60	»	10	R	MM	R	R
450 gr+7 gr+5 gr/10 kg	Gambas	51,00		5	R	M	R	R
450 gr/10 kg	Gambas	55,01		10	R	M	R	R
400 gr/10 kg	Gambas	47,12		5	R	M	R	R
gr/10 kg	Chorizo blanco	109,29	26,05	0	R	R	R	B
gr/10 kg	Gambas	41,64	10,12	0	B	B	B	B
gr/10 kg	Gambas	46,05	11,05	0	B	B	B	B
gr/10 kg	Gambas	—	Descompp.	0	R	MM	M	M
425 gr/10 kg	Gambas	—	»	0	R	MM	M	M
375 gr/10 kg	Gambas	—	»	0	R	MM	M	M
370 gr/10 kg	Gambas	—	»	1	B	B	B	B
850 gr/20 kg	Quisquillas	—	—	10	B	B	B	B
800 gr/20 kg	Quisquillas	—	—	10	MB	MB	MB	MB
700 gr/20 kg	Quisquillas	—	—	0	MB	MB	MB	B
1200 gr/30 kg	Chorizo Rojo	61,15	14,17	0	MB	MB	MB	MB
400 gr/10 kg	Quisquillas	92,17	7,25	0	B	R	B	R
900 gr/30 kg	Chorizo Rojo	41,70	27,15					

Los ensayos 11, 12 y 13 se realizaron en el barco «Queipo de Llano», los dos primeros con metabisulfito potásico y el n.° 13 con metabisulfito sódico, siendo los días de conservación de estas experiencias 16 días para la n.° 11 y 14 días para las de 12 y 13. El ensayo n.° 11 se realizó con «Chorizo blanco» no apreciándose síntomas de melanosis después de 16 días, siendo calificada la prueba de regular en las restantes apreciaciones organolépticas debido a que los crustáceos estaban algo reblandecidos. Las experiencias 12 y 13 se realizaron con gambas, apreciándose que después de 14 días de conservación (12 a bordo y 2 en cámara) no había síntomas de melanosis y fueron calificadas de buenas las restantes características organolépticas. Es de hacer notar que las cajas de estas experiencias tenían hielo abundante y que antes de ser examinadas se conservaron dos días en una cámara a 2-4°C.

Los ensayos 17, 18 y 19 se realizaron a bordo del barco «Jumar» empleando metabisulfito potásico, con tiempos de conservación a bordo de 14, 13 y 12 días respectivamente. En el examen organoléptico se

apreció un 1, 10 y 10 % de melanosis debido probablemente a irregularidades en la distribución del producto, siendo las restantes apreciaciones organolépticas calificadas de Bueno y Muy Bueno. Las cajas correspondientes a estas experiencias contenían también suficiente hielo.

Los ensayos 20 y 22 se realizaron a bordo de los barcos «Virgen de la Caridad» y «Verdel» con «Chorizo rojo» y empleando metabisulfito potásico. No se apreció melanosis en las dos experiencias después de 15 y 14 días respectivamente. Siendo calificadas las restantes características de Bueno y Muy Bueno excepto el olor y la textura del ensayo 22 que se dieron de Regular debido principalmente a que algunos «Chorizos» tenían aplastadas las cabezas (los del fondo de la caja).

El ensayo n.º 21 se efectuó en el barco, con bodegas climatizadas, «Apóstol San Andrés» con quisquillas y empleando metabisulfito potásico. Después de 17-18 días de conservación a bordo no se apreciaron síntomas de melanosis y siendo las restantes calificaciones del examen organoléptico de Muy Buenas.

RESUMEN Y CONCLUSIONES

Se han realizado 28 experiencias a bordo de barcos congeladores con soluciones de diversos productos químicos al objeto de prevenir la melanosis que se produce en los crustáceos congelados y en la manipulación posterior que sufren las cajas (de unos 10 kilos) para su aplicación a la venta al público o cocido. De los productos ensayados, han dado excelentes resultados el metabisulfito potásico, hidrosulfito sódico y metabisulfito sódico. Empleando soluciones de estos compuestos del 3 al 7 % y sumergiendo los crustáceos en estas soluciones de 2 a 5 minutos se previene efectivamente la melanosis de los crustáceos congelados y se pueden mantener, una vez descongelados, 48 horas en hielo sin que aparezcan síntomas de melanosis (tablas I y II). Este período de tiempo de 48 horas nos parece más que suficiente para efectuar las manipulaciones necesarias para la comercialización de los crustáceos congelados. Los contenidos en SO_2 residual de estos ensayos han oscilado entre 4,15 y 18,10 mg de SO_2/100 gr, porcentajes que quedan muy por debajo de los límites admitidos.

En barcos de conservación por hielo se han efectuado 31 ensayos; 9 empleando soluciones y bañando los crustáceos y 22 espolvoreando los productos sólidos directamente.

En las 9 experiencias que se han realizado sumergiendo los crustáceos en soluciones antes de ser colocados en hielo, se ha empleado únicamente metabisulfito potásico e hidrosulfito sódico. De los resultados especificados en la tabla n.º III se ve que se previene bastante bien el en-

negrecimiento de los crustáceos empleando soluciones de estos compuestos del 6,5 al 15 % con tiempos de inmersión de 3 a 15 minutos. No obstante, en tiempos superiores a los 9 días de conservación a bordo, se ha apreciado una tendencia al reblandecimiento de las cabezas de los «Chorizos».

De los productos empleados en las 22 experiencias realizadas por espolvoreo en los barcos de conservación por hielo sólo han dado resultados positivos aquellos en los que se empleó metabisulfito potásico y sódico. Muchos de los ensayos realizados no pueden ser tenidos en cuenta (barcos «V. Caridad», «Navarra» y «María Cordero», Tabla IV) ya que las cajas experimentales a su llegada a puerto estaban totalmente faltas de hielo, no apreciándose, sin embargo, síntomas de melanosis ni aún después de 19 días de conservación a bordo. Los crustáceos de estas experiencias se encontraban en avanzado estado de descomposición incluidas pruebas con sólo 7 días de conservación (tabla IV, ensayo 10).

En los ensayos realizados a bordo de los barcos «Queipo de Llano», «Jumar», «V. de la Caridad» (marea del 22-11-66) y «Apóstol San Andrés» se obtuvieron resultados muy buenos después de 14, 15 y 17 días de conservación a bordo, siendo de notar que todas estas experiencias estaban cuidadosamente hechas, teniendo las cajas suficiente hielo durante la travesía.

A la vista de lo expuesto anteriormente, llegamos a la conclusión de que no existe problema alguno en la prevención de la melanosis de los crustáceos congelados ya que bañando a éstos en soluciones de metabisulfito potásico o sódico o hidrofulfito sódico se conservan perfectamente sin la aparición de síntomas de melanosis aún después de mantenerlos, una vez descongelados, 48 horas en hielo. Con respecto a la conservación por hielo llegamos a la conclusión que para tiempos superiores a los 12-14 de conservación de los crustáceos a bordo de los barcos, es necesario que las cajas tengan durante toda la travesía hielo suficiente, siendo casi imprescindible para ello que los barcos tengan bodegas climatizadas. Esto quedó demostrado en los ensayos realizados a bordo del barco «Apóstol San Andrés», en el cual una caja de «Quisquillas» tratada con metabisulfito potásico, después de 17-18 días a bordo, no presentaba síntomas de melanosis siendo calificadas las restantes apreciaciones organolépticas de Muy Bueno.*

* Con posteridad a la redacción de este trabajo varios barcos congeladores han preparado cantidades considerables de crustáceos empleando metabisulfito potásico que creemos de interés el reseñarlas. Así el barco «Consuelo Veiga» (salida 9-1-68) y (llegada el 29-6-68) preparó 6500 kg de gambas y 1000 kg de chorizos, sumergiendo a éstos en soluciones de metabisulfito potásico al 5 % durante 5 minutos. Estas partidas llegaron en perfectas condiciones y fueron comercializadas normalmente, teniendo incluso mayor aceptación por parte de los compradores los crustáceos tratados de esta forma, debido principalmente a un mejor colór. El 14-7-68 llegó un cargamento de unos 69 000 kg de «Chorizo rojo» y «Chorizo blanco» trata-

AGRADECIMIENTO

Queremos hacer constar nuestro agradecimiento por la colaboración prestada a la realización de este trabajo a los señores R. Garrido y R. Cabrerizo, inspectores provinciales de Sanidad Veterinaria de Cádiz y Huelva respectivamente, a D. M. Veiga, presidente de la Cooperativa de Armadores de Buques de Pesca de Cádiz, a D. B. Marquínez, presidente del Grupo Autónomo de Buques de Pesca de Altura de Huelva, a D. J. Gestoso, patrón mayor de la Cofradía de Pescadores de Cádiz y a los armadores, patrones y contramaestres de los barcos donde se realizaron las experiencias.

Asimismo agradecemos a los señores A. Fernández, de «Industrias Gaditanas del Frío, S. A.», E. Quintana, de «Crustáceos de Andalucía» y D. J. Sibón, armador de buques de pesca, por la ayuda prestada.

SUMMARY

The prevention of melanosis of freezing and ice-stored crustaceans using various dips and solids chemicals compounds is studied.

The sodium and potasium metabisulfite dip and the sodium hidrosulfite dip were very effective in the protection against melanosis (black spot) through period of storage in the freezer trawlers (Table I and II).

Fresh crustaceans treated on the traelers with solid potasium and sódium metabisulfite and stored in comercial ice, delayed deterioration and also avoid totally the development of melanosis.

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dos con metabisulfito potásico, de la misma forma que el anterior, procedentes del barco Capitán Emilio, que también llegaron en perfectas condiciones. Asimismo el 16-9-68 se descargaron unos 57 000 kg de gambas, «Chorizo rojo» y «Chorizo blanco» procedentes del barco congelador «Andes». Estos crustáceos también fueron tratados con solución al 5 % de metabisulfito potásico y llegaron en perfectas condiciones.

The Journal of Biochemistry, Vol. 52, No. 5, 1962

The Deoxygenation of Dilute Oxyhemoglobin by Sodium Dithionite

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(Received for publication, June 13, 1962)

It has been known that obscure side reactions occur and interfere accurate kinetic measurements with oxyhemoglobin and hemoglobin when oxyhemoglobin is deoxygenated by addition of sodium dithionite ($Na_2S_2O_4$) (1—6). Since these reactions are rapid and transient, it is hard to observe the spectra of intermediate compounds which are formed during the deoxygenation by sodium dithionite. Dalziel and O'Brien (7) gested that hydrogen peroxide or some other oxidation products aerobically generated in the reaction with $Na_2S_2O_4$ may be responsible for these side reactions.

Recently, two kinds of hemoglobin have been partially purified from body-wall tissue of *Ascaris lumbricoides* in this laboratory (8). Those were distinct from mammalian hemoglobins in that the rate of deoxygenation by $Na_2S_2O_4$ was slower and that the reduced hemoglobin combined with cyanide, forming a stable-cyanide ferrous hemoglobin complex (8, 9). By use of this unusual hemoglobin, the obscure side reactions in the deoxygenation of oxyhemoglobin by $Na_2S_2O_4$ could be followed by the authors more precisely than was done with horse hemoglobin by Dalziel and O'Brien (7). These observations are revealed in this communication.

Of oxyhemoglobin solutions of *Ascaris* prepared according to the method previously described, the fraction A_1 (8) has been used in this experiment. Spectral measurements were made with a Cary model 11 recording spectrophotometer. To buffered oxyhemoglobin solution was added a small amount of solid $Na_2S_2O_4$ and the mixture was quickly covered with liquid paraffin to avoid contact with air.

Formation of Methemoglobin prior to the Formation of Deoxygenated Hemoglobin—Fig. 1 shows the spectral changes at the Soret region which were recorded immediately after addition of $Na_2S_2O_4$ to dilute oxyhemoglobin solution. It

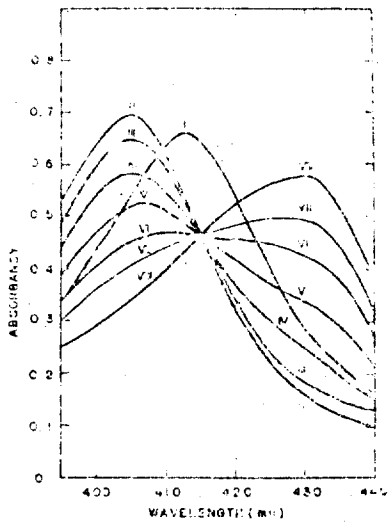

Fig. 1. Spectra recorded during deoxygenation of oxyhemoglobin from body-wall tissue of *Ascaris lumbricoides* with $Na_2S_2O_4$ in 0.1 *M* sodium phosphate buffer, pH 7.0, at 23°C. Curve I is for a oxyhemoglobin solution containing 5.5×10^{-5} *M* as heme concentration. Curve II was recorded immediately after the addition of $Na_2S_2O_4$. Curves III through VIII were recorded at 116, 141, 206, 296, 396 and 810 seconds after addition of $Na_2S_2O_4$.

appears from the results that oxyhemoglobin (Soret band; 412 mμ) was converted to a compound absorbing at 405 mμ and then de-

oxygenated hemoglobin was formed from this 405 mμ compound. The spectrum at 14 minutes after mixing was of a typical deoxygenated hemoglobin which has a peak at 431 mμ. An isosbestic point occurred at 415 mμ. The maximum yield of deoxygenated hemoglobin was attained in 14 minutes under the condition indicated in the figure. The rate of these spectral changes was dependent on the pH. It was rapid in acid solution and slow in alkaline solution. Practically, below pH 6.5 only the formation of deoxygenated hemoglobin was observed but not the 405 mμ compound.

Since hydrogen peroxide or some unstable oxidation products are generated by the addition of $Na_2S_2O_4$ to a solution (*10*), it is suggested that they are responsible for the appearance of 405 mμ compound prior to the formation of deoxygenated hemoglobin, and that the compound may be methemoglobin which has a peak at 405 mμ.

The suggestion is supported by the results represented in Fig. 2. Fig. 2 shows the spectral changes at the Soret region which were recorded in the deoxygenation by $Na_2S_2O_4$ in the presence of cyanide. The result indicates that the compound absorbing at 417 mμ, corresponding to the Soret band of cyanide-methemoglobin of *Ascaris* (*8*), is formed prior to the formation of cyanide-ferrous hemoglobin complex which has a peak at 428 mμ. An isosbestic point occurs at 420 mμ. At low concentration of cyanide (below 1×10^{-3} *M*) the cyanide-ferrous hemoglobin complex was not formed, but the reaction terminated in the formation of deoxygenated hemoglobin after transient appearance of 417 mμ compound (*9*).

It is conceivable from these observations that one of the side reaction of deoxygenation of oxyhemoglobin by $Na_2S_2O_4$ is the transient formation of methemoglobin by hydrogen peroxide or by some oxidation products aerobically generated by $Na_2S_2O_4$.

Formation of an Unknown Compound from Deoxygenated Hemoglobin—Absorbancies of deoxygenated hemoglobin at 431 mμ and of cya-

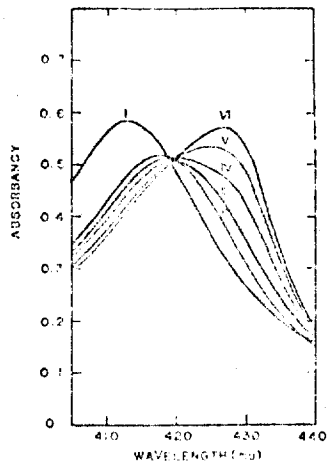

Fig. 2. Spectra recorded during deoxygenation of oxyhemoglobin with $Na_2S_2O_4$ in the presence of potassium cyanide, 3.3×10^{-1} *M*, in 0.1 *M* sodium phosphate buffer, pH 8.0, at 23°C. Curve I is for a oxyhemoglobin solution containing 5.0×10^{-5} *M* as heme concentration. Curve II through VI were recorded at 1, 5, 15, 81 and 135 minutes after mixing. The completion of this reaction has been attained approximately at 6 hours.

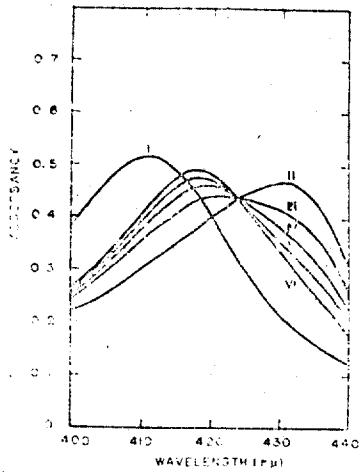

Fig. 3. Spectral changes recorded after deoxygenation of oxyhemoglobin with $Na_2S_2O_4$ in 0.1 *M* sodium phosphate buffer, pH 6.0, at 23°C. Curve I is for a oxyhemoglobin solution of 4.4×10^{-5} *M* (as heme concentration) which slightly contains methemoglobin. Curve II was recorded immediately after addition of $Na_2S_2O_4$. Curve III through VI were recorded at 10, 25, 50 and 70 minutes after reduced hemoglobin has been formed.

nide-ferrous hemoglobin at 428 mμ were stable at pH 7.0 or at slightly alkaline condition. But when the hemoglobin solution was allowed to stand for a few hours at slightly acid condition, it was observed that absorbancies at 431 mμ or at 428 mμ gradually decreased and a stable compound absorbing at 417 mμ was formed. An isosbestic point occurs at 424 mμ during the course of the change (Fig. 3). As seen in Fig. 3, the absorbancy of deoxygenated hemoglobin at 431 mμ was reducing to form a compound absorbing at 417 mμ at 10 minutes after mixing, while at pH 7.0, the absorbancy at 431 mμ was increasing to form deoxygenated hemoglobin at that time, indicating that the formation of 417 mμ compound is favoured by acid conditions. The 417 mμ compound observed in Fig. 3 differed from cyanide-methemoglobin (Fig. 2) and also from methemoglobin-hydrogen peroxide complex observed by Dalziel and O'Brien (*11*) which has absorbancy maxima at 417, 545 and 582 mμ, in the fact that it was stable, the absorbancy at 417 mμ is independent on the amount of added $Na_2S_2O_4$ and the isosbestic points did not occur at 420 mμ observed in Fig. 2.

The chemical nature of the 417 mμ compound in Fig. 3 remains as yet obscure, and the compound does not correspond to any of the decomposition products of hemoglobin so far reported.

This work was supported by a Grant for Scientific Research from the Ministry of Education.

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ORIGINAL PAPERS

SODIUM HYDROSULFITE.

By FREDERICK W. HEYL and FRANK E. GREER.*

There exists a voluminous literature [1] concerning derivatives of hydrosulphurous acid ($H_2S_2O_4$) and its reduction product sulfoxylic acid (H_2SO_2), but it is confined almost exclusively to the patent literature. Neither of these is known in the form of the acid. The sodium salt of the former and the sodium salt of formaldehyde sulfoxylic acid ($CH_2OH.OSO\ Na$) are important commercial reducing agents. The preparation and stabilization of these and their varied use in textile printing and dyeing is the subject of several hundred patents.

With the discovery by Ehrlich and Bertheim [2] of the reduction of arsanilic acid to p. amino-phenyl arsenoxid, and to diaminoarsenobenzol by means of sodium hydrosulfite this reagent becomes of interest beyond the textile field. In fact it is a reducing agent capable of very general use, having been successfully employed twenty years ago [3] for the reduction of certain nitrophenols.

Sodium formaldehyde sulfoxylate, aside from its value as a reducing agent, has the power of condensing with aromatic amines [4] to form soluble neutral derivatives of the amines, but at the same time producing marked alteration in the pharmacological nature of the original base, as *e. g.* in neoarsphenamine. This reaction is usually represented as follows:

$$X.NH_2 + HO.CH_2.\ OSO\ Na \rightarrow X.NH\ CH_2OSO\ Na.$$

This condensation of aromatic amines with sodium formaldehyde sulfoxylate gives a product which will not reduce indigo-carmine in

*Holder of The Upjohn Coöperative Fellowship at Kalamazoo College (1920-1921). This paper is based upon the thesis presented by Mr. Greer to the Faculty of Kalamazoo College, in partial fulfillment of the requirements for the degree of Master of Science.

[1] Jellinek, *Das Hydrosulfit*, 2 Vol. (1911).

[2] B. 43, 917 (1910).

[3] Chem. Zent. 1, 1014 (1900).

[4] Reinking, Dehnel, Labhart B. 38, 1069 (1905).

the cold [5], although some reduce indigo in the cold if acid is present. According to Binz and Marx [6] the condensation between the salts of the bases and sodium formaldehyde sulfoxylic acid proceeds in two phases.

(1) R. NH_2HCl + HO CH_2O SO Na → R.NH. CH_2 OSOH.

(2) R. NH. CH_2 OSOH + R. NH_2. HCl → R.NHCH_2 $OSONH_3$. R.

There is thus formed an insoluble ammonium salt. In the preparation of neoarsphenamine there is obtained from the reaction mixture small amounts of material insoluble in sodium carbonate. This material probably represents substances of the salt type. (2) In any event the detailed study of this substance requires eminently pure sulfoxylate, and the use of commercial sulfoxylate through the presence of impurities further complicates the study of the reaction.

In the same way the production of arsphenamine requires proportionately large quantities of hydrosulfite for the reduction of 3-nitro-4hydroxyphenylarsinic acid. According to the Ehrlich process, 263 g. would require 3930 g. commercial sodium hydrosulfite (80%)

$$2(NO_2)\ (OH)\ C_6H_3\ As\ O(OH)_2 = 10\ Na_2S_2O_4.$$
$$263 \qquad\qquad = 870$$

or about 3.6 times the theoretically required amount. It appeared to us that the chemical variability of the product might be due not so much to the arsinic acid, which is prepared analytically pure, but rather to the reagent which is not only impure, containing unknown substances but which is known to react in a secondary manner to produce organic sulphur compounds in variable quantities.[7]

It was therefore desirable to review the patent literature and arrive at a laboratory method for the production of these reagents. The object of this paper is to describe the production of these substances and their properties. We obtained most satisfactory products by first producing crude sulfoxylate by D. R. P. 256, 460. (1913). This was purified by recrystallization. From pure sulf-

[5] Reinking, B., 38, 1074 (1905).
[6] B., 43, 2344 (1910).
[7] *J. Chem. Soc.* 117, 370 (1920).

oxylate, the anhydrous hydrosulfite was prepared by the reaction discovered by Bazlen.[8]

CH_2OH OSONa + 2 $NaHSO_3$ → $Na_2S_2O_4$ + HCHO. $NaHSO_3$.

The original experiment however leads to the highly unstable crystalline hydrate $Na_2S_2O_4$. $2H_2O$, but by modification to the extent of carrying the reaction out at a temperature of 70° (above the transition point, 52°) the anhydrous salt is precipitated by salting out.[9]

While this is not as economical as the direct production of hydrosulfite by the reduction of bisulfite with zinc, it will be found to be better adapted for the laboratory scale as the product requires no purification whatever, and is free from zinc, and is invariably permanent.

EXPERIMENTAL.

Preparation of Sodium Formaldehyde Sulfoxylate.—This is produced smoothly according to D. R. P. 256, 460, the most essential requirement being violent mechanical agitation. 312 g. $NaHSO_3$ (3 mol.), 257 g. formalin (90 g. CH_2O) are mixed with the addition of 54 cc. water. Water (500 cc.) is used to further dilute the reaction mixture and to moisten the reducing mixture consisting of zinc dust (360 g.) and zinc oxide. (150 g.) This is gradually added in small portions and the reduction mixture is held at 70°. After the addition of the reduction mixture, the reaction is continued for two hours at 100-105°, with violent agitation. The mixture is filtered and the precipitate washed with hot water. This filtrate concentrated to dryness yields the commercial product.

When the filtrate was concentrated to 400 cc. a crop (A) weighing 347 g. separated. This was redissolved in warm water and fractionated, yielding a top fraction of 56 g. which separated from the warm solution. It had a purity of only 6.2%. The next crops; 22 g., 45 g., and 48 g. were pure sulfoxylate (99%). The fifth crop separating from the warm solution weighed 11 g. and was only 24% sulfoxylate. The sixth crop weighed 33 g. and was 74.4% pure.

The filtrates were joined and concentrated and from the warm solution 20 g. of 16% material was removed by filtration. Then 2

[8] B. 38, 1065 (1905).
[9] U. S. P. 990, 457 (1911).

crops of 59 g. and 51 g. separated. The purity of these was 96% and 81% respectively. The yield of sulfoxylate is about 60%.

For laboratory purposes it is somewhat simpler to reduce the addition product of formaldehyde and commercial hydrosulfite.[10]

($NaHSO_2$. CH_2O $NaHSO_3$. CH_2O).

The use of zinc and acetic acid as there directed leads to the presence of acetates and renders the subsequent purification by crystallization more difficult. The following is a practical laboratory process: 400 g. hydrosulfite is dissolved in 400 cc. formalin and 300 cc. water. This is reduced with a mixture of zinc dust (300 g.) and zinc oxide (100 g.), first at 70° and then two hours at 100-105° and with agitation. To the liquid a few drops of sodium carbonate solution are added and the filtrate on concentration always yields a crop of almost pure sulfoxylate. By fractionation yields of 70% are obtained.

Calc. for $NaHSO_2$. CH_2O. C = 10.1 : H = 2.5. Found: C = 9.4, 10.0, H = 2.7, 2.55.

In recrystallizing this substance it is desirable to keep the solutions alkaline with Na_2CO_3 and to have a small amount of formaldehyde present. The temperature is never allowed to exceed 70° at which temperature this substance is exceedingly soluble. (1 liter dissolves 500-600 g.[11]) Otherwise there is a constant decomposition with the emission of a garlic or mercaptan-like odor.

Analysis of Sodium Formaldehyde Sulfoxylate.—CH_2OH OSO Na $2H_2O$.

This substance is now procurable in a rather high state of purity (85%) on the American market. It may be analyzed readily by the direct titration of a hot solution (acidified with acetic acid) using standardized methylene blue solution. The titrations were carried out in an atmosphere of CO_2. The solution is warmed gradually during the titration and not boiled until the end point is reached. In a well conducted titration an excess of methylene blue solution is had at all times until almost at the end point. The titration liquid is taken from the flame just as the color is about to vanish, before each new addition of methylene blue. Reduction does not proceed rapidly until a temperature of about 50-60° is attained.

[10] D. R. P. 165, 807.

[11] Osann, B. 38, 2290 (1905).

Baumann, Thesmar and Frossard[12] analyze sodium formaldehyde sulfoxylate by titrating with an ammoniacal copper sulfate solution in a stream of hydrogen at 55°. This method is satisfactory for routine purposes but does not give results quite as high as the methylene blue procedure.

$$\underset{154}{NaHSO_2.\ CH_2O.\ 2\ H_2O} = \underset{2}{2\ H} = 2\ CuSO_4\ 5\ H_2O.$$
$$2(249.6)$$

it is advisable to prepare an N/4 solution by dissolving 62.4 g. crystalline copper sulfate in water, adding 200 cc. conc. ammonia water, and making the volume to one liter. Then 1 cc. = 0.01925 g. crystalline sulfoxylate. It is our practice to weigh 0.385 g. of the sample, whence each 0.1 cc. of the $CuSO_4$ solution used is equivalent to 0.5% sulfoxylate, and a sample requiring 20 cc. is 100% pure.

When sulfoxylate is titrated with iodine in neutral solutions a quantity of iodine equivalent to four atoms iodine is required. This is an unsatisfactory method for the analysis of crude products as the impurities are likewise oxidized. Baumann, Thesmar and Frossard have made a distinction between sulfoxylate and sodium formaldehyde bisulfite. They showed that while the latter is not oxidized by iodine in neutral solution it may be in alkaline solution. Hence in this common mixture the end point is first recorded for the sulfoxylate in neutral solution, whereupon the partially titrated fluid is rendered alkaline with sodium bicarbonate and a second volume of iodine is slowly required by the bisulfite compound, but the end point is not sharp.

A sample of analytically pure sodium formaldehyde sulfoxylate (calc: H_2O = 23.4. Found, 23.8) was analyzed in the anhydrous state. (Calc: Na, 19.5: S, 27.12. Found: Na, 19.5 S, 26.75).

0.1174 g. required 39.62 cc. iodine (1 cc. = 0.01283 g. I).
0.1500 g. required 18.75 cc. methylene blue sol. (1 cc. = 0.0058 g. Fe).
0.385 g. required 19.4 cc. N/4 copper sulfate sol.

These results correspond to a purity of 100.7, 99.7, and 97% respectively and indicate the fact that in general the copper titration gives slightly low results.

[12] Rev. Gen. Mat. Color 8, 354 (1904).

Solubility of Crystalline Sulfoxylate.—The crystalline product loses its water of crystallization completely when dried in a vacuum over calcium chloride or phosphorous pentoxide. The solubility of the crystalline substance in glycerine was determined.

25 cc. of the saturated solution (18°) weighed 34.2967 g. 5 cc. of this was diluted to 100 cc. and of this 5 cc. was titrated with methylene blue solution. Found: Original 5 cc. = 2.5329 g. sulfoxylate. Therefore 100 cc. of the saturated solution contains 86.5 g. gylcerine plus 50.66 g. sulfoxylate. Therefore 100 cc. glycerine (sp. grav. 1.262) will dissolve about 74 g.

In methyl alcohol the solubility is much lower. 50 cc. of a saturated solution weighs 42.5613 g. By titration 100 cc. contains 8.30 g. sulfoxylate and 76.73 g. methyl alcohol; 100 cc. methyl alcohol (sp. gr. 0.8) will dissolve about 8.8 g. sulfoxylate.

When aqueous solutions of sodium formaldehyde sulfoxylate are treated with barium chloride solutions, either neutral or with one mol. of sodium hydroxide a slight precipitate of the barium salt separates. (3.5% from 5% solutions.) Calcium chloride gives no precipitate in neutral solutions. Both chlorides precipitate sodium sulfite almost completely under the same conditions. Sodium formaldehyde bisulfite is very slightly precipitated by barium chloride, but not by calcium chloride in neutral solutions, but by increasing the alkalinity the baryta forms an insoluble barium salt. Thus in the presence of 0.3 mole sodium hydroxide 67% of the formaldehyde bisulfite was precipitated by barium chloride.

Effect of Sulfoxylate on Rats.—A series of intravenous injections into white rats gave the following results:

Dose milligrams per kilo.	Weight of rat.	Vol. of solution, cc.	Time Seconds.	Result.
400	146	0.58	50	Lived
600	190	1.14	50	"
800	119	0.95	60	"
1000	172	1.72	80	"
1200	93	1.12	65	"
1400	123	1.72	85	"

This chemical is apparently tolerated in very large doses, no disturbance being noted.

Sodium Hydrosulfite.—Analysis of Commercial Samples.

Some time ago a number of samples purchased as hydrosulfite were examined and it was found that some confusion exists concerning this substance. The products consisted in some cases of anhydrous $Na_2S_2O_4$, having a high reducing power on methylene blue, at room temperatures. Other products appeared to consist entirely of sodium formaldehyde sulfoxylate, exhibiting no reducing power under these conditions whilst still other samples had an intermediate reducing power. The last named were either deteriorated products or mixtures.

After studying several methods we finally adopted this method of Knecht and Hibbert[11] for routine work. For this purpose standard solutions of titanous chloride, and of methylene blue are prepared, the former being standardized against ferrous ammonium sulfate.

$$2\,Ti\,Cl_3 = 2\,H = 2\,Fe = Na_2S_2O_4 = C_{16}H_{18}N_3SCl$$
$$2 \times 154.5 = 2 \quad 112 \quad 174 \quad 373.75$$

An approximately one per cent. solution is prepared by diluting 50 cc. of the commercial 20% Ti Cl_3 solution with 50 cc. of conc. hydrochloric acid, boiling and diluting with air-free acidulated water to one liter. This solution is stored under special precautions under hydrogen.

For purposes of standardization 0.7 g. Mohr's salt is dissolved in 25 cc. diluted sulphuric acid, and titrated with a very slight excess of potassium permanganate solution. Into this solution is now run the titanous chloride solution until the ferric salt has been just reduced, using KSCV as an outside indicator.

This titanous chloride solution is only moderately stable. For instance, in one case we found 1 cc. $TiCl_3$ sol. = 0.006 g. Fe (January 10). Later (March 25) 1 cc. = 0.00565 g. Fe.

For the relative standardization 25 cc. of the mythelene blue solution is diluted with about 25 cc. air-free water, acidified with 5 cc. of 25 per cent. acetic acid. The titration is made in an Erlenmeyer flask fitted with a two-holed stopper, one of which is an inlet for a stream of carbon dioxide. We prepared the methylene blue solution so that it will be equivalent to the titanous chloride. It is perhaps advisable to permit a small amount of insoluble material to separate if necessary and to use the supernatent solution. This solution is stable, only an occasional comparison with the standard titanous chloride solution being necessary.

For the analysis of commercial hydrosulfites, since 1 cc. 1% $TiCl_3$ sol. = 0.003625 g. Fe = 0.005631 g. $Na_2S_2O_4$, we usually use for analysis 0.12-0.14 g. anhydrous hydrosulfite if we have 1% titanium chloride solution. This is placed in a dry Erlenmeyer, covered with 25 cc. (an excess) standard methylene

[11] B., 40, 3827 (1907). Knecht and Hibbert, "New Reduction Methods in Volumetric Analysis." Longmans, Green & Co., 1918.

blue solution, all the air is displaced with CO_2 and then the solution is acidified with 5 cc. of 25% acetic acid, and after standing at room temperature a short time the excess of methylene blue, which the $Na_2S_2O_4$ has failed to reduce, is titrated with the titanous chloride solution.

Example: 0.1648 g. sodium hydrosulfite + 24.8[14] cc. standard methylene blue solution, required for decolorization, 9.3 cc. $TiCl_3$ solution. (1 cc. $TiCl_3$ sol. = 0.00588 g. Fe = 0.009135 g. $Na_2S_2O_4$. In a duplicate assay 0.1584 g. required 10 cc. $TiCl_3$. Found, 85.9% and 85.3%.)

The following results were obtained upon commercial samples submitted as "hydrosulfite."

Taken.	Found.	Per Cent. Purity.
0.1200	0.1016	84.7
0.103	0.0809	78.5
0.104	0.0073	6.98
0.107	0.0082	7.17
.102	0.0	0.0
.103	0.0103	10.12
.102	0.0828	81.2
.103	0.0061	5.91

The samples showing low reduction contain formaldehyde and are used for vat indigo dyeing. These should not be listed as hydrosulfites. The reduction of methylene blue was materially increased by raising the temperature, indicating the presence of sodium formaldehyde sulfoxylate.

Purification and Stability of Sodium Hydrosulfite.—We endeavored to ascertain the possibility of increasing the reducing power *i. e.*, increasing the purity. Aqueous solutions are unstable. In order to determine the rate of deterioration, quantities of $Na_2S_2O_4$ (85.3%) were weighed into a dry 20 cc. volumetric flask, covered with toluene, 1 cc. of 2N sodium hydroxide solution was added and the solution was brought to the final volume with air-free water. The solution was emptied into a small burette, which had been filled with carbon dioxide, and contained a layer of toluene. This solution was titrated against 10 cc. of a standardized acidified methylene blue solution in an atmosphere of carbon dioxide. 10 cc. methylene blue = 0.0453 g. $Na_2S_2O_4$.

[14] Twenty-five cc. of the solution = 24.8 cc. $TiCl_3$ solution.

$Na_2S_2O_4$ (85.3%) Taken.	Volume.	Solution Used for 10 cc. M.B.	Time.	Loss %
0.1036	20	10.5	At once	—2.3
0.1050	20	10.16	"	—2.6
0.1040	20	10.15	"	—2.3
0.0707	20	15.07	"	—0.3
0.1513	20	7.15	"	—1.8
0.1026	20	10.7	10 min.	—3.2
0.1031	20	10.62	20 "	—.3.0
0.1177	20	9.6	6 hrs.	—6.0
0.1062	20	13.7	22½ "	—27 0
0.1064	20	16.75	16 " (no toluol)	—40

The above solutions were alkaline and a conspicuous breakdown is observed after 5 hours.[15] The protection of toluol is an important factor. All of the above titrations were made in acetic acid solutions. The titrations give the same results in neutral or alkaline media, but the solutions are turbid (errors —3.5 and 2.2%). Even when rapidly performed the results obtained fall below those obtained by using an excess of methylene blue solution by 2.5%. For rapid work the method is sufficiently accurate and the titanium chloride solution can be dispensed with. This method is like the original Bernsthen[16] method, using indigo carmine and is quite neat. Knecht & Hibbert point out that this method is not satisfactory for certain commercial samples, since they decompose with the evolution of SO_2 when dissolved in water; they claim furthermore that if alkali is added to preserve the solution that oxidation proceeds so rapidly that within a "few seconds" the results are vitiated As shown above, with careful work the error approximates = 2.5%.

We attempted to purify commercial samples of hydrosulfite as follows: 50 g. high grade hydrosulfite was poured upon 125 cc. air-free water, containing a few cubic centimeters of sodium hydroxide solution at 70° in a current of carbon dioxide or under toluene. The solution was filtered through asbestos in a closed system under carbon dioxide and the filtrate cooled in an ice bath under an inert gas. After crystallization was complete (1 hour), the crystalline hydrosulfite was filtered in a closed system, and mixed upon the alundum

[15] See Lumiere, Lumiere & Seyewetz, *Bull. Soc. Chim. 33*, 931 (1905).

[16] Bernsthen & Drews, B. 13, 2283 (1880), Schützenberger & Risler, *Bull. Soc. Chim.* 19, 152 (1873).

filter with absolute alcohol. The suspension was transferred to a flask containing 300 cc. of alcohol and sodium (15 g.) ethylate, or methylate with mechanical stirring. The dehydrated product was filtered in a closed system and washed[17] with alcohol and ether. The funnel was transferred to a desiccator containing carbon dioxide and dried in vacuo over phosphorous pentoxide. The highest purity obtained was 83.5 per cent., so that practically nothing was gained, although some insoluble sludge is removed by filtering solutions of the commercial product.

This experiment is difficult to manipulate, and even if the preliminary work is successfully conducted, high-grade recrystallized products are prone to decompose upon opening the desiccator. One sample which had been dried for two weeks at 15 to 20 mm., and which assayed over 75 per cent., burst into flame while a sample was being assayed. Some samples showed a tendency to warm up, and were returned to a vacuum in various stages of decomposition.

The ease with which this decomposition proceeds was amply demonstrated in the above-mentioned series of experiments, many of which miscarried. This difficulty certainly justifies the many efforts toward this goal, which are found in the patent literature. D. R. P. 267,872[18] (1912) directs the mixing of the hydrosulfite with an excess of aniline and evaporation in a vacuum to dryness, with uninterrupted stirring. The object of this procedure is to avoid filtration of the crystalline salt, but the product must of course be impure. In subsequent patents the conditions of the distillation are altered.[19] In other patents[20] evaporations are conducted with alcohol, xylol, hydrocarbons, ammonia, and alkalin substances.

In D. R. P. 280,181, zinc hydrosulfite is mixed with dried sodium acetate, and the mixture is extracted with 94-96 per cent. alcohol, which dissolves the zinc and sodium acetates and leaves a residue of anhydrous sodium hydrosulfite.

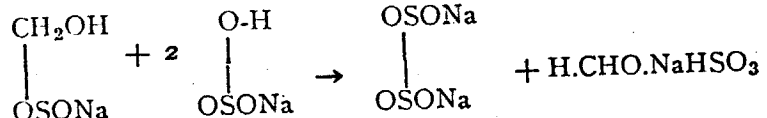
Bazlen in his paper on the composition of hydrosulphurous acid, points out the ease with which these alkali salts oxidize, and also how readily thiosulfate is formed even in the absence of air, and he states that drying in a vacuum at ordinary temperature or at more elevated ones in the absence of air leads to decomposition. His method for dehydration consists in placing an alcoholic magma in a Soxhlet and extracting for several hours, using lime in the receiver.[21] He reports an analysis on the pure salt, $Na_2S_2O_4$.

It was with these patents in mind that we attempted the above purification, but the results were such that we were led to attempt purification and preparation through the use of another principle, *i. e.*, salting out the anhydrous form. The temperature above which

[17] U. S. P. 987,170.
[18] U. S. P. 1,156,107 (1915).
[19] D. R. P. 279,389; U. S. P. 1,207,782 (1916).
[20] D. R. P. 213,586, 188,139, 223,260, 207,593; U. S. P. 861,218.
[21] D. R. P. 160,529 (1904).

the anhydrous form cannot exist is 52°. According to D. R. P. 171,991 (1905), one saturates a concentrated solution of sodium hydrosulfite (50-70°) with sodium chloride, or one may warm crystalline sodium hydrosulfite at 50-70° under saturated brine. Thus 2000 volumes concentrated hydrosulfite solution is heated to 50-70° and 510 parts of salt are added with stirring. When the characteristic separation of the anhydrous form is complete, it is filtered in a closed system, using CO_2, washed with alcohol and dried in a vacuum over sulfuric acid. This method is very satisfactory on the laboratory scale, but it does not essentially raise the purity of the commercial material, although the patent claims that 100% material results. This probably depends upon the nature of the impurity in the original material.

Preparation of Sodium Hydrosulfite on Laboratory Scale.—In our last effort to secure analytically pure, anhydrous hydrosulfite we attempted to avoid the impurities originally present in the hydrosulfites, by preparing it from analytically pure sodium formaldehyde sulfoxylate. Bazlen proved that hydrosulfite can be prepared by the interaction of sodium formaldehyde sulfoxylate and sodium bisulfite. He permitted the crystalline form to separate. U. S. P.



990,457 (1911) combines the principles of this reaction with that of the salting out process as follows: 420 cc. of bisulfite solution containing 208 g. $NaHSO_3$, containing no free sulfurous acid are heated to 60°; 140 g. salt are introduced and the temperature raised to about 65°. In a second vessel a concentrated solution of 118 g. sodium formaldehyde sulfoxylate (230 cc.) is heated to 65°. The contents of both vessels are mixed as rapidly as possible. There is a rise of about 8° and a heavy precipitate of anhydrous hydrosulfite separates. We filtered this off in a closed system under CO_2 washed with alcohol, ether, and immediately placed the product in a CO_2 desiccator and dried at 15-20 mm. The purity claimed for this product, *i. e.*, "an amount of the salt containing one gram of sulfur reduces 4 g. of indigo" is equivalent to a 97.7% purity.

Our yields on small scale experiments in the quantities above outlined give yields of 55 to 60% of theory, and a purity of 80-85%. The product is stable when kept in tightly stoppered containers. Larger scale production would probably result in higher purity.

Toxicity of Sodium Hydrosulfite.—The failure to obtain products by the above process having a purity of at least 95% is a matter of considerable surprise. In the presence of air the damp salt oxidizes as follows:

$$Na_2S_2O_4 + O \rightarrow Na_2S_2O_5 \rightarrow Na_2SO_3 + SO_2$$
$$Na_2S_2O_4 + O + H_2O \rightarrow 2\ NaHSO_3$$

In the absence of air it is said to decompose forming pyrosulfurous and thiosulfuric acids. We have never observed any con-

$$2\ Na_2S_2O_4 \rightarrow Na_2S_2O_3 + Na_2S_2O_5$$

spicuous alteration in well made samples. It is however a fact that the oxidation products of the dry substance are quite as toxic as the hydrosulfite itself.

The following results were obtained: A preparation having a purity of about 83% was dissolved in water and quantities amounting to 150, 180, 210 mg. per kilo proved not fatal, and no effects were observed. With a dose of 240 mg. per kilo death ensued in five minutes.

A second sample having approximately the same purity (83.5%) gave the following results: The rats lived at doses of 100 and 125 mg. per kilo, but at 150 mg. per kilo, death ensued in 7 minutes.

A few similar tests were made upon some of the partially decomposed samples that were obtained when it was attempted to dehydrate the crystalline product with sodium ethylate. The toxicity is not proportional to its reducing activity as is shown by the following results. A badly decomposed sample, analyzing 28% failed to kill in doses of 50, 100 and 150 mg. per kilo, but at 200 mg. per kilo death took place in four minutes, and 10 minutes respectively. This sample is almost as toxic as the above mentioned 83% material.

A sample which had run down to 65% in the desiccator over P_2O_5, suddenly evolved clouds of SO_2 when the desiccator was opened. The analysis showed only 14% (reducing power). When this was injected the rats lived at 200, 300, 325 mg. per kilo, but were killed at 350 mg. (4 minutes).

From the above tests it will be observed that the dry decomposition does not proceed simply from a toxic to a nontoxic substance, nor, is the physiological test, a method for determining the degree of decomposition.

Small doses (25-50 mgs. per kilo) of sodium hydrosulfite injected intravenously into white rats produce no reaction. As the dose (100 mgs. per kilo) is increased the rat shows signs of labored breathing within a few seconds of the beginning of the injection. This quickly wears off and usually within five or ten minutes the rat is apparently normal. Still higher doses (150 mgs. per kilo) produce a more labored breathing with evidence of suffocation. When the maximum sub-lethal dose is reached the rat shows symptoms immediately. Dyspnea becomes acute and the rat doubles up with convulsions within three to five minutes. After eight or ten minutes it has completely recovered and moves about normally. There are no delayed symptoms in rats which survive the injection. The toxic dose produces the same immediate symptoms which rapidly progress in intenisty until death which usually occurs within five to ten minutes.

We wish to acknowledge the biological assistance of Mr. Payne.

Contribution From the Chemical Research Laboratory,
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Biochem. J., 47: 43-52, 1950

Some Observations on the Kinetics of Haemoglobin in Solution and in the Red Blood Corpuscle

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(*Received* 5 *May* 1949)

The kinetics of the rapid reactions of oxygen and carbon monoxide with the oxygen-carrying pigments have been studied by the rapid-flow methods (Hartridge & Roughton (1923*a*–*c*, 1925, 1927), Millikan (1932, 1933*a*, *b*, 1936) and Roughton (1934*a*–*d*)). For myoglobin (Mgb), Millikan found that the rate of combination of oxygen could be expressed by the equation

$$\frac{d[O_2Mgb]}{dt} = k'[O_2][Mgb] - k[O_2Mgb], \qquad (1)$$

and furthermore that $k/k' = K$, the equilibrium constant of the reaction ($K = [O_2][Mgb]/[O_2Mgb]$) as determined from the dissociation curve, which is in this case a rectangular hyperbola. These simple relations are to be expected on the basis of the Law of Mass Action, since the myoglobin molecule only contains one iron atom and thus reacts reversibly with one molecule of oxygen according to the equation

$$O_2 + Mgb \rightleftharpoons O_2Mgb. \qquad (2)$$

In the case of mammalian haemoglobin, the situation is much more complicated, since the molecule contains four iron atoms and reacts reversibly with four molecules of O_2 or CO. It is true that Hartridge & Roughton found that their kinetic results could, within the limits of error and range of their measurements, be described by an equation similar to (1), namely

$$\frac{d[\mathrm{XHb}]}{dt} = k'[\mathrm{X}][\mathrm{Hb}] - k[\mathrm{XHb}], \qquad (3)$$

where [X] = concentration of dissolved O_2 or CO; [XHb] = concentration of oxyhaemoglobin or carboxyhaemoglobin in g. mol. of combined O_2 or CO/L.; [Hb] = concentration of reduced haemoglobin expressed in the same units as XHb.

The equilibrium results are, on the other hand, generally interpreted by some form of Adair's (1925) intermediate compound hypothesis according to which

$$\frac{y}{100} = \frac{[\mathrm{XHb}]}{[\mathrm{XHb}]+[\mathrm{Hb}]} = \frac{K_1 p + 2K_1K_2p^2 + 3K_1K_2K_3p^3 + 4K_1K_2K_3K_4p^4}{4(1 + K_1p + K_1K_2p^2 + K_1K_2K_3p^3 + K_1K_2K_3K_4p^4)}, \qquad (4)$$

where y = percentage saturation; p = pressure of X; K_1, K_2, ..., are the respective equilibrium constants of the reactions $Hb_4 + O_2 \rightleftharpoons Hb_4O_2$, $Hb_4O_2 + O_2 \rightleftharpoons Hb_4O_4$, etc.

Some attempts have recently been made to reconcile equations (3) and (4) (Wyman, 1948; Roughton, 1949), but there is clearly need of much further work in this field, both theoretical and experimental. One great difficulty is that many different sets of values of K_1, K_2, K_3, K_4 give a good fit between equation (4) and the experimental results, though, even so, it always appears necessary to assign a much higher* value to K_4 than to any of the other constants. This means that the last O_2 (or CO) molecule combines with much greater affinity than the preceding ones. Pauling (1935) has suggested that a similar but smaller 'interaction' occurs in the case of the lower intermediates, but the equation derived on his detailed hypothesis has recently been shown not to be valid for the best available dissociation curves (Roughton, 1943, 1949; Wyman, 1948; Roughton, Paul & Longmuir, 1949).

Several kinetic points arise out of these and other considerations:

(*a*) Since $K_4 = k_4'/k_4$, where k_4' and k_4 are the velocity constants of the reaction

$$Hb_4O_6 + O_2 = Hb_4O_8,$$

then if K_4 is relatively large, either k_4' must also be relatively large or k_4 relatively small. Calculation of the empirical velocity constants, k' and k, of equation (3) should therefore, at high values of y, show a rising trend in k' or a falling trend in k, or perhaps both. No such tests have hitherto been made above $y = 75\%$: in this paper analysis of present and earlier data shows that both these effects do in fact occur.

(*b*) In all previous reaction-velocity work, the haemoglobin before mixture has been either fully reduced or fully saturated. Tests should be made as to whether the results obtained with partially saturated haemoglobin as the starting point are in agreement: a discrepancy would indicate that when haemoglobin is partially saturated, the concentrations of the various intermediates, or their energy levels, differ appreciably according to whether the haemoglobin is in equilibrium or is in a state of change when the particular partial saturation occurs. Such observations might also throw light upon a peculiar result of Roughton (1934*d*), who found that, at pH 10 and 15°, CO combines twice as rapidly with haemoglobin just after its formation from oxyhaemoglobin by admixture with sodium dithionite ($Na_2S_2O_4$) as it does if the reduced haemoglobin is more than 2 sec. old. It is possible that the 'freshly' reduced haemoglobin might not have been quite completely reduced and that small traces of Hb_4O_2, Hb_4O_4, etc., might still have been present. In such an event, CO would have reacted with iron atoms adjacent to some which were already combined with O_2, this leading perhaps to a faster rate of combination than in the case of 'old' reduced haemoglobin in which no Hb_4O_2, etc., could have been present.

In this paper, tests are given on the effect of initial partial saturation of the haemoglobin, both on the rate of combination of CO with haemoglobin and on the rate of dissociation of oxyhaemoglobin in presence of $Na_2S_2O_4$, these two reactions being the most satisfactory for studies of this kind. Adult sheep haemoglobin was used, since it was the main source of material used in previous work, and furthermore it is not believed to split into submolecules, at the concentrations suitable for our experiments. The results were mostly negative, except perhaps in the dissociation of oxyhaemoglobin at neutral pH. Here, however, an interesting new phenomenon was discovered, the oxyhaemoglobin in the presence of $Na_2S_2O_4$ often appearing to dissociate in two phases, one fast and the other slow. The explanation is probably complex, and though not fully worked out, it has been felt worth-while to discuss the data in some detail, in view of the widespread use of $Na_2S_2O_4$ for reduction of oxyhaemoglobin and other substances of physiological interest.

(*c*) Recently, Nicolson & Roughton (1949) have made a much fuller analysis than was possible in Roughton's earlier paper (1932), of the rates of reaction of O_2 and CO with haemoglobin in red-cell suspensions as compared with the rates with haemo-

* Work now in progress by one of us (F. J. W. R.) suggests that the ratio of K_3 to K_4 may be higher than previously supposed.

globin in homogeneous solution. They believe that it is possible, by their methods, to compute approximately for the first time, the permeability of the red-cell membrane to O_2 and CO. For this purpose, new and more accurate data were required on the rates of the reactions of haemoglobin in solution and in the red cell. Such data were secured as a by-product of the main experimental projects of this paper, and a brief description of the actual experiments is accordingly included.

EXPERIMENTAL METHODS

General principle

The reagents are prepared in separate bottles (2·5–3·0 l. capacity) and driven thence, by compressed N_2 or air, through separate leads into a mixing chamber. From there the mixed fluid emerges into a glass observation tube, and the concentration of the various haemoglobin compounds in the streaming fluid at various points along the observation tube are determined by Millikan's (1932) differential photocolorimetric method (slightly modified). Such determinations, together with a knowledge of the rate of fluid flow down the observation tube and of the total concentration of each reagent, give the data required for measuring the velocity of the reaction.

Experimental details

Mode of preparation of the reagents

(*a*) *Oxygen-free water or buffer solutions.* A bottle was usually half-filled with solution, evacuated and rolled vigorously for 4 min. Nitrogen was then admitted to atmospheric pressure, the gas phase re-evacuated and the rolling repeated. Alternatively, the bottle was warmed to about 30°, evacuated until the solution boiled, rolled, re-evacuated and rolled again without admission of N_2.

(*b*) *Solution of* CO. The solution was first freed of O_2 as in (*a*). CO was then admitted to a suitable pressure and the bottle rolled therewith for 4 min. (or longer if the bottle was more than half full of liquid). The CO was either prepared from Na formate and conc. H_2SO_4, or drawn from a cylinder of compressed gas: in either case it was stored under pressure over alkaline $Na_2S_2O_4$, so as to remove traces of CO_2 and of O_2.

(*c*) *Solution of* O_2. The bottle was partially filled with solution as in (*a*), the air evacuated off, replaced with a suitable pressure of O_2 and rolled for 4 min. or longer. The concentration of dissolved O_2, or of CO in (*b*), was calculated from the composition of the gas phase, its pressure, the relative volumes of gas and liquid phases and the appropriate solubility coefficients.

(*d*) $Na_2S_2O_4$ *solution.* The standard concentrations used were 0·1 and 0·2 %, and were prepared by dissolving weighed amounts of the solid in the buffer solutions with minimal contact with air. $Na_2S_2O_4$ in solution develops acidity, and appropriate amounts of NaOH (as determined by the glass electrode) were also added to the buffers, so as to restore the latter to the stated pH values.

(*e*) *Reduced haemoglobin solution.* Defibrinated sheep blood was reduced by repeated evacuation and shaking while warm, as described by Forbes & Roughton (1931), the whole process taking 30–60 min. The required volume of reduced blood was then transferred anaerobically, via a mercury reservoir and burette, to the bottle containing O_2-free water, the final haemoglobin concentration being usually about 0·4 %, at which strength adult sheep haemoglobin shows no tendency to dissociate into submolecules. It was not found satisfactory to prepare reduced haemoglobin merely by adding $Na_2S_2O_4$, since, as previous work has shown, there is danger, on prolonged standing, of formation of variable amounts of choleglobin (Legge & Lemberg, 1941) and possibly of other compounds.

(*f*) *Oxyhaemoglobin solution.* This was prepared in several ways: (i) by adding a suitable volume (usually 1 in 30) of defibrinated sheep blood to aerated water; (ii) the reduced blood, prepared as in (*e*), was re-aerated by 15 min. rotation in a tonometer with air and then added to the aerated water; (iii) the reduced blood was added to 29 parts of water, previously equilibrated with O_2 at a partial pressure of 300 mm. Hg, the dissolved O_2 content of the water being sufficient to re-oxygenate the haemoglobin almost completely. The purpose of methods (ii) and (iii) was to control for possible changes in the haemoglobin which might occur during the reduction process described under (*e*).

(*g*) *Carboxyhaemoglobin solution.* This was only required for calibration purposes and was prepared by adding 1 part of defibrinated sheep blood, which had been rotated for 15 min. with CO at 1 atmosphere pressure, to 29 parts of water.

The special reagents required for the experiments on the red-cell suspensions are described in the section dealing with the red-cell results.

The reaction-velocity apparatus

The reagent bottles were connected via a tube of 5 mm. bore, with a tube of capillary bore in parallel, to either side of a two-way Perspex mixing chamber. The rate of flow could thus be varied about threefold, according to whether the 5 mm. bore tube or the capillary tube was open. The observation tube was first of the form shown in Fig. 1*A*, but later when more extended times of reaction were required, other forms (Fig. 1*B*, *C*) were used. Type *B* proved unsatisfactory for red-cell suspensions, since a rotatory motion was imparted to the fluid when it entered the enlarged part of the tube, thus resulting in a partial sedimentation of the red cells, easily visible in optical irregularities as the fluid left the enlarged part of the tube. The form of fluid motion in type *C* did not cause such irregularity. The driving pressures ranged from 20 to 40 cm. Hg and the rate of flow down the observation tube from 30 to 150 cm./sec. The mixing chamber and observation tube were attached to a vertical adjustable stand, by means of which different parts of the observation tube could be brought under examination.

Differential photocolorimetry

Light from a 6 V. 12 W. projection lamp, fed by two 2 V. accumulators in series (for greater stability the lamp was thus run below its maximum brightness) was focused on to the observation tube. The beam then passed through a red-green filter, as described by Millikan (1932), and thence on to an EEL differential selenium cell (Evans Electroselenium Ltd. Harlow, Essex). The photocurrent generated by the latter was recorded by a Pye moving-coil galvanometer (deflexion 450 mm./μa. at 4 m. scale distance, time of

indication about 1·5 sec.). At each position of the observation tube, the light beam was so focused and the red-green filter so adjusted that (i) the galvanometer deflexion was as near zero as possible (small residual deflexions being balanced off by a compensating potentiometer), (ii) the change from oxyhaemoglobin (or carboxyhaemoglobin) to reduced haemoglobin caused a deflexion of 50–120 mm. A reading was then taken with reduced haemoglobin flowing through the tube, then with the reacting fluid, and finally with oxyhaemoglobin or carboxyhaemoglobin standard. Errors due to drifts in light intensity and/or photocell sensitivity are thus much reduced.

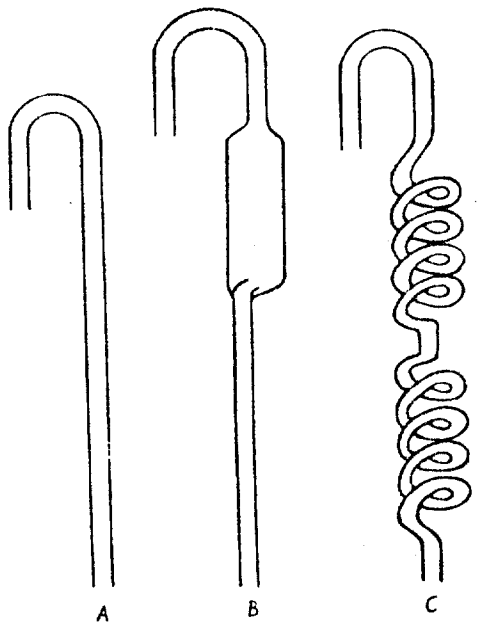

Fig. 1. Various forms of observation tube in this paper. *A*, general form; *B*, form for special study of processes with a rapid phase and slow phase; *C*, form for study of red-cell suspensions.

Under our conditions, as in Millikan's, the proportions of the various haemoglobin compounds present (i.e. the 'percentage saturation') were obtained by linear interpolation from the end-point readings. That this procedure was correct to within 1–2% saturation was checked, both by Millikan's theoretical method and also by means of haemoglobin solutions of known percentage saturation.

RESULTS AND DISCUSSION

1. *The rate of combination of dissolved carbon monoxide with reduced haemoglobin*

A. *Haemoglobin solutions.* In Fig. 2*A*, *B* is shown the rate of combination of a solution of CO in water (0·22 mM) with (*a*) fully reduced Hb (0·25 mM), and (*b*) the same haemoglobin containing initially 19% COHb. The haemoglobin solution was made up of 1 part blood + 29 parts of 0·1 M-ammonium chloride – 0·05 M-ammonia buffer mixture, pH approx. 9·5, temp. 17°. The rates of reaction do not differ materially provided allowance is made for the higher total concentration of CO, dissolved and combined in (*b*).

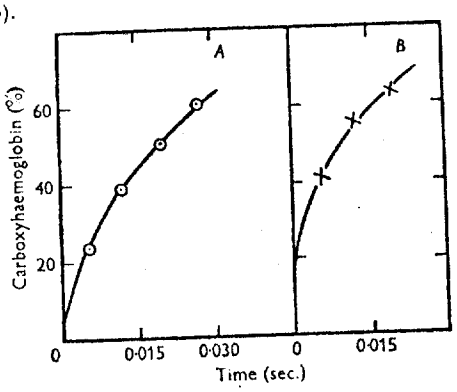

Fig. 2. *A*, rate of combination of CO with reduced Hb; *B*, rate of combination of CO with 19% COHb + 81% reduced Hb.

If α = total concentration of CO, dissolved and combined, β = total concentration of haemoglobin expressed in gas-combining capacity of O_2 or CO, z = concentration of COHb at time t, then equation (3) may be written

$$\frac{dz}{dt} = l'(\alpha - z)(\beta - z) - l, \qquad (4)$$

the constants k' and k being replaced by l' and l, so as to distinguish the process as a CO reaction. The

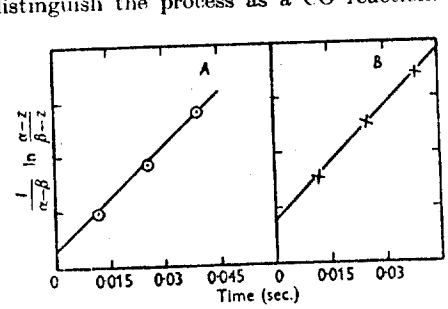

Fig. 3. Plot of $\frac{1}{\alpha-\beta}\ln\frac{\alpha-z}{\beta-z}$ versus time. *A*, for data of Fig. 2*A*; *B*, for data of Fig. 2*B*.

term, lz, can be neglected until almost the end of the reaction, and equation (4) on integration then gives

$$l't = \frac{1}{\alpha-\beta}\ln\frac{\alpha-z}{\beta-z} + \text{constant}.$$

Fig. 3*A*, *B* shows the plot of t versus $\frac{1}{\alpha-\beta}\ln\frac{\alpha-z}{\beta-z}$ respectively for the experiments of Fig. 2. The points

fall within experimental error on straight lines of the same slope in the two cases. A similar result was obtained in a duplicate experiment at pH 9·5, and also in a pair of experiments at pH 6·8 (0·05 M-phosphate buffer, temp. 16–17°). At the more alkaline pH the reduced haemoglobin is almost completely ionized, and at the more acid pH almost completely unionized, as regards the oxylabile group (pK approx. 8) whose ionization is affected by combination with O_2 or CO, but in both forms the simple equations appear equally applicable. The experiments of Fig. 2 indicate that the reactivity of partially saturated haemoglobin to CO is the same whether the system is in equilibrium or is in a state of change, and thus give no help as to the reason for faster combination of CO with 'new' haemoglobin, referred to in the introduction. No rise in l' is to be seen in the later stages, but this was scarcely to be expected since the reaction was only followed to about 60 % of completion. Fig. 4, however, shows the plot of $\frac{1}{\alpha-\beta}\ln\frac{\alpha-z}{\beta-z}$ against t for 10 points obtained in a previously published experiment by Bateman & Roughton (1935, fig. 2). In this case the rate of combination of CO with haemoglobin was followed to about 85 % of completion, by two independent but concordant methods (thermoelectric and photoelectric). The first four points, which just span the first half of the reaction, are seen to fall satisfactorily on a straight line, as in all previous such data. But the latter points clearly lie well above the line and indicate that in the later stages the calculated value of l' tends to increase to 1·4 or so times its initial value. An increase of this order is to be expected on the development of the intermediate compound theory, considered elsewhere by Roughton (1949).

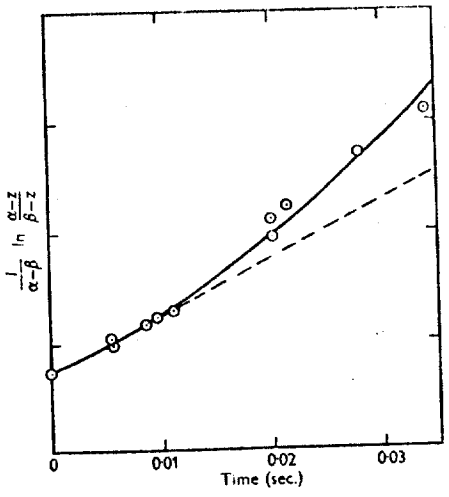

Fig. 4. Plot of $\frac{1}{\alpha-\beta}\ln\frac{\alpha-z}{\beta-z}$ versus time for rates of CO combination as given by data of Bateman & Roughton (1935).

B. *Comparison of haemoglobin solutions and red-cell suspensions.* The previous experimental data used by Roughton (1932) in his theoretical investigation were defective in several respects. (i) The solutions and suspensions were not prepared from the same source of blood, though it is known that the Hb-reaction rates show appreciable scatter between individuals of the same species (Table I; Roughton, 1932). (ii) The determinations of haemoglobin saturation were made with the reversion spectroscope, which was not only less accurate than the present photoelectric technique, but was, furthermore, not available in the early phases of the process (i.e. saturation 0–30 %), wherein the theoretical treatment is most reliable. (iii) Most of the data were obtained on O_2 uptake, which is physiologically of much more interest than CO uptake: from the theoretical point of view, however, the latter process is more satisfactory, in view of its slower rate and absence of appreciable back reaction. We therefore decided to fill in these gaps, by doing several experiments on the rate of CO uptake by haemoglobin solutions and red-cell suspensions, both prepared from the same sheep blood.

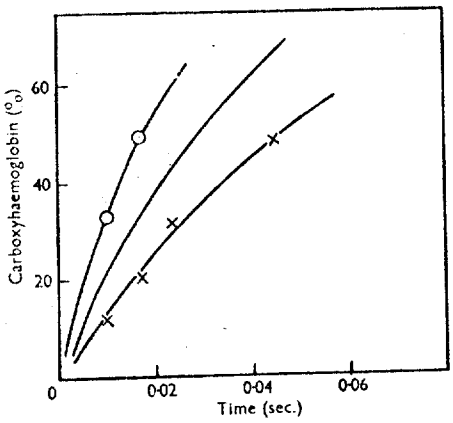

Fig. 5. Rate of combination of CO with reduced Hb solution and with reduced corpuscle suspensions of ram and pregnant ewe blood. Hb solution (ram, ewe), ⊙—⊙; ram cell suspension, ——; pregnant ewe cell suspension, ×—×.

The red-cell suspensions were prepared by adding 1 part of blood to 29 parts of Ringer-Locke solution (9 g. NaCl, 0·42 g. KCl, 0·24 g. $CaCl_2$, 0·2 g. $NaHCO_3$ l.). The suspensions were periodically stirred (with minimal air contact) so as to keep them uniform throughout the experiment. For the haemoglobin solutions the blood was laked by adding 14 parts of distilled water, and then 15 parts of double

strength Ringer-Locke solution. The CO solution was made by shaking Ringer-Locke solution with CO at appropriate pressures, as described above for CO buffer solutions. Four experiments were done altogether, two on ram blood and two on blood from pregnant ewes (kindly supplied by the late Sir Joseph Barcroft).

Fig. 5 shows that the rates of CO uptake by the ram and ewe haemoglobin solution are the same, but considerably faster than the rates by the corresponding cell suspensions, which differ from one another, the rate of the ram cell suspension being about 1·6 times faster than the rate of the ewe cell suspension. This interesting difference was confirmed in other experiments of the series, and is believed to be mainly due to a much lower permeability of the pregnant red-cell membrane. The theoretical interpretation of these results is under investigation by Nicolson & Roughton (1949), and some of their preliminary conclusions have already been published (Legge, Nicolson & Roughton, 1949), though these require modification in view of the recent determinations of the diffusion coefficients of CO and N_2 in 35–40 % (w/v) haemoglobin solutions (Roughton *et al.* 1949).

2. *The rate of dissociation of oxyhaemoglobin in presence of* $Na_2S_2O_4$

A. *Haemoglobin solutions.* Hartridge & Roughton (1923*c*) found that the rate of dissociation of oxyhaemoglobin in presence of $Na_2S_2O_4$ was independent of the concentration of the latter, provided this exceeded a minimal value (approx. 0·1 %). They therefore supposed that a two-stage process takes place, viz:

$$\text{Oxyhaemoglobin} = \text{haemoglobin} + \text{dissolved } O_2, \qquad (a)$$

$$O_2 + Na_2S_2O_4 \rightarrow \text{oxidation products of } Na_2S_2O_4, \qquad (b)$$

and that when the limiting $Na_2S_2O_4$ concentration was exceeded, reaction (*b*) proceeded so fast and completely that O_2 is kept negligibly small. Under these circumstances the rate of recombination of dissolved O_2 with haemoglobin in reaction (*a*) would also be negligible, and the overall rate of dissociation of oxyhaemoglobin gave the speed of reaction (*a*) from left to right without appreciable back reaction. The rate of dissociation of oxyhaemoglobin, so observed, was found to follow a unimolecular course from 80 % O_2Hb downwards. Millikan's more accurate work led to the same conclusion, and his experiments on the rate of dissociation of oxyhaemoglobin and oxyhaemocyanin in presence of $Na_2S_2O_4$ gave added support to the original mechanism which has for some time been generally accepted. There are, however, still some points in regard to the speed and order of reaction (*b*) which need to be settled if the whole process is fully to be understood.

Fig. 6 shows the results of one of two concordant experiments at pH approx. 10, 0·1 % $Na_2S_2O_4$, on the rate of dissociation when the haemoglobin was initially (i) 99–100 % saturated, (ii) 50–60 % saturated. The points in the latter case fall, within experimental error, on the same curve as in the former case. The curve is logarithmic between 70 and 0 % O_2Hb, and in this range the kinetics of the reaction thus conform to the equation

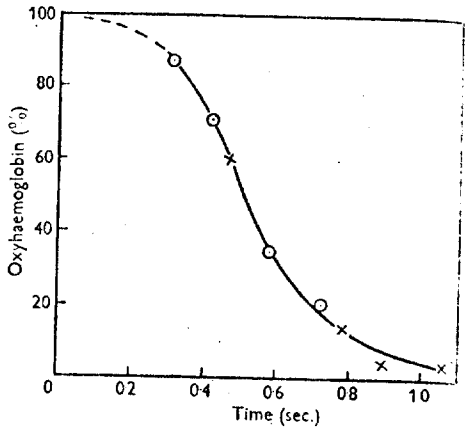

Fig. 6. Rate of dissociation of O_2Hb in presence of $Na_2S_2O_4$ at pH 10, temp. 11°. ⊙ = 100 % O_2Hb at start; × = 60 % O_2Hb at start.

$$\frac{-d[O_2Hb]}{dt} = k[O_2Hb].$$

Between 100 and 80 % O_2Hb the curves of this paper show a lag period, as do also Millikan's curves (1932), when closely inspected. This lag has hitherto been attributed to reaction with the $Na_2S_2O_4$ of the excess of dissolved O_2, which is initially present just after mixture (i.e. at about 75 mm. pO_2, where pO_2 = partial pressure of O_2) and has to be reduced to a low value (e.g. about 10 mm. pO_2) before reaction (*a*) can occur to an appreciable degree. The lag period, however, continues further than would be expected if this were the whole explanation. This point may be illustrated by considering in more detail the four points in the experiment of Fig. 6, in which the O_2Hb started by being 99–100 % saturated. The actual numerical data of y and t are shown in Table 1,

Table 1. *Determination of velocity constant for rate of dissociation of oxyhaemoglobin over various time intervals*

Percentage saturation (y)	Time in sec. (t)	k
100	0	
87	0·31	0·42
71	0·42	1·85
35	0·58	4·4
20·4	0·72	3·9

together with the calculated values of k over various time ranges. The value of k is seen to rise to a plateau, but does not reach it until y has fallen to 70 %. Consider the range between $y = 87$ % and $y = 71$ %. At $y = 87$ % the equilibrium p_{O_2} at the temperature of the experiment, 11°, would be 2·7 mm., and at $y = 70$ % it would be 2·0 mm. The maximum amount of physically dissolved O_2 which the $Na_2S_2O_4$ would have to remove over this range would only be $\frac{2{\cdot}6 - 2{\cdot}0}{760} \times 0{\cdot}037$ (= solubility coefficient of O_2 in water) = 0·000034 ml./ml. The combined O_2 which is removed on the other hand amounts to

$$\frac{87 - 71}{100} \times 0{\cdot}003 \text{ (= gas-combining capacity of Hb sol.)}$$
$$= 0{\cdot}00048 \text{ ml./ml.}$$

As the latter figure is 14 times greater than the former, it seems impossible that the low value of k in the range 87–71 % O_2Hb should be appreciably due to the dissolved O_2 effect. It must, in fact, be in the main due to a genuine kinetic feature of the dissociation of O_2Hb, as is indeed to be expected on the theory put forward by Roughton (1949). This feature had hitherto escaped notice, partly owing to the paucity of dissociation rate data above $y = 80$ % and partly through being obscured by the dissolved O_2 effect, the importance of which is undoubted between $y = 100$ % and $y = 95$ % (about), but fades out very rapidly when y falls below 95 %.

Fig. 7 shows the results of one of two concordant experiments at pH 6·8. In these cases, owing to the greater speed of the reaction, there are not sufficient data in the upper range for a decision as to an initial lag period. Both experiments showed a rather faster initial rate of dissociation in the case of the partially saturated haemoglobin than would be expected from the curve for the fully saturated haemoglobin. The effect is, however, overshadowed by a more remarkable finding, namely that the dissociation appears to proceed in two phases, a rapid one from 100 to about 30 % O_2Hb, and then a slow one from 30 to 0 % O_2Hb. This latter phase takes several seconds to reach completion, as was shown by following the progress of the change when the running fluid was suddenly stopped. Human haemoglobin at pH 6·0–7·4 (Fig. 2; Millikan, 1936) shows a similar diphasic effect, which is brought out clearly when the logarithm of O_2Hb concentration is plotted against time. The discrepancy in the rates of the two phases is, however, not so great as in Fig. 7. Perhaps this was why Millikan did not notice or comment on the effect. Later in this section the matter is investigated and discussed further.

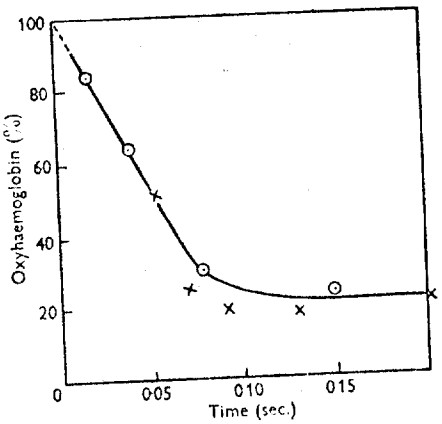

Fig. 7. Rate of dissociation of O_2Hb in presence of $Na_2S_2O_4$ at pH 6·8, temp. 15·8°. ⊙ = 100 % O_2Hb at start; × = 51 % O_2Hb at start.

B. *Comparison of haemoglobin solutions and red-cell suspensions.* Roughton (1932) found that the rate of dissociation of oxyhaemoglobin in sheep red-cell suspensions was about one-quarter of the rate in homogeneous solution. A similar ratio is suggested by Dirken & Mook's (1931) data on ox haemoglobin, though Millikan's results (1932) for human haemoglobin indicate a rather lower figure. In nearly all this earlier work, however, the pH of the interior of the red cells was probably not the same as that of the haemoglobin solutions, and part of the observed difference in the dissociation rates might be due to difference of pH, which has a large effect *per se*.

We have tried to obviate this difficulty by working at the isoelectric point of haemoglobin (pH approx. 6·8), for here there is practically no difference in pH between the interior of the red cells and the surrounding liquid.

Table 2. *Comparison of rate of dissociation of oxyhaemoglobin in red-cell suspensions and haemoglobin solutions* (*ram*)

Ram no.	pH	Temp. (°)	Half time of dissociation (sec.) (*a*) Hb solution	(*b*) Cell suspension	(*a*)/(*b*)
1	6·8	15	0·03	0·14	0·21
2	6·8	13·5	0·03	0·16	0·19
	7·1	13·5	0·05	0·21	0·23
3	6·8	14	0·03	0·12	0·25
	7·1	14	0·03	0·17	0·18

The red-cell suspension, the haemoglobin and the $Na_2S_2O_4$ were therefore all made up in Ringer-Locke solution buffered with a bicarbonate-CO_2 mixture of pH 6·8–7·1. The procedure was thus the same as in section 1, save that the $NaHCO_3$ concentration in the Ringer-Locke solution was increased from 0·8 g. l. to 4·1 g./l., and all the solutions were finally equilibrated with CO_2 at pressures of 150–300 mm. Hg. The final concentrations in the mixed fluid were 1 g. $Na_2S_2O_4$ and 1 part of blood in 60.

Table 2 summarizes the results of comparisons carried out with blood from three different runs. The mean ratio, at pH 6·8, for the rate in cells to the rate in solution is 0·22, and is about the same at pH 7·1, thus confirming the results of the earlier experiments. The mathematical interpretation of the present data is also under investigation by Nicolson & Roughton (1949).

C. *Further study of the apparent diphasic dissociation of oxyhaemoglobin at neutral* pH. This observation aroused our interest for several reasons. (i) It might have some fresh bearing on the nature of the reaction between oxygen and haemoglobin, and in particular might provide new evidence that one of the four haem groups in the molecule behaves differently from the other three. (ii) It might be related to Roughton's (1934*d*) unexplained observation that when dilute sheep oxyhaemoglobin, between pH 6 and 7, is suddenly mixed with O_2-free water, the oxyhaemoglobin dissociates in two stages, about two-thirds of the oxygen coming off in about 0·5 sec. and the remainder over a period of several seconds. (iii) In the circulating blood *in vivo* it is often observed that the venous blood contains appreciable reserves of oxygen even when the whole body, or particular organs of it, are in a state of severe oxygen want. It seemed possible that a slow final phase in the rate of oxyhaemoglobin dissociation might be a factor concerned in this effect.

We therefore carried out some thirty experiments altogether in the hope of elucidating the matter further. Unhappily the results were irregular and it was only in the latter weeks of our collaboration that we got satisfactory clues as to the sources of the varying effects.

In fifteen experiments at pH 6·8 (0·05 M-phosphate buffer, temp. 9–19°) a break was found in every case, but the percentage saturation at which the break occurred (referred to as the 'plateau') varied from 3 to 30 % O_2Hb. Three to five tests were also made at each of the following pH values: 6·2, 7·1, 7·4 and 7·7 (all in phosphate buffer) and at pH 6·8 and 7·1 in CO_2-bicarbonate buffer. In the three tests at pH 6·2, the plateau occurred at a much lower percentage O_2Hb than with the same haemoglobin solution at pH 6·8, but at the other pH values there was no marked difference from the results at pH 6·8.

In three out of four experiments on the cell suspensions (in CO_2-bicarbonate buffer, pH 6·8–7·1), no distinct plateau was seen at all and in the fourth one, only a rather low 'plateau' at about 9 % O_2Hb. There thus appeared to be a genuine difference between the haemoglobin solutions and the corpuscle suspensions, and since $Na_2S_2O_4$, and its products of oxidation, are not believed to permeate the red-cell membrane, or at most only very slowly, it seemed that the diphasic effect might be associated with some action of $Na_2S_2O_4$ or its oxidation products on the haemoglobin. In a control experiment in which the $Na_2S_2O_4$ concentration was varied two-fold, the diphasic course of the reaction was not affected: this suggests that the products of reaction of $Na_2S_2O_4$ with O_2, rather than the $Na_2S_2O_4$ itself, are responsible since their concentration (under constant haemoglobin conditions) would hardly be altered when the $Na_2S_2O_4$ concentration is halved or doubled.

Further support for this view was obtained along the following lines. In the majority of the later tests the mixture of O_2Hb and $Na_2S_2O_4$, which had run through the apparatus, was allowed to stand for 15 sec. and then slowly siphoned back through the observation tube, so as to serve as the source of reduced haemoglobin for calibration purposes at each observation point. This standard may be referred to as reduced haemoglobin *A*. Two other forms of reduced haemoglobin standard were also used on various occasions. In both cases a solution of completely reduced haemoglobin (prepared as described in the section on experimental methods) was mixed in the reaction apparatus with the $Na_2S_2O_4$ solution and either collected and similarly siphoned back through the observation tube (= reduced haemoglobin *B*) or readings were taken on the mixed fluid as it travelled down the observation tube before collection (= reduced haemoglobin *C*). In certain of the later experiments* the reading given by reduced haemoglobin *A* differed appreciably (in the sense of appearing to be more reduced) from that given by reduced haemoglobin *B* or *C*, and in such cases a high 'plateau', of the order of 20 % O_2Hb was seen. On the other hand, in five experiments in which no appreciable difference in reading between reduced haemoglobin *A* and *B* (and/or *C*) was observed, the 'plateau' was always low, i.e. not more than 10 % O_2Hb. Our general conclusion was thus that in certain cases the oxidation products of $Na_2S_2O_4$ produced abnormal haemoglobin pigments, and when this happened a much more pronounced, apparent 'plateau' was found in the rate of dissociation of the oxyhaemoglobin.

* In one case reduced haemoglobin standard *A* was also made from blood dissolved in oxygenated water (pO_2 = 730 mm. Hg) as well as from the usual blood dissolved in aerated water, (pO_2 = 150 mm. Hg). The oxygenated Hb on mixing with the $Na_2S_2O_4$ gave a standard which appeared, photocolorimetrically, to be distinctly more 'reduced' than the aerated Hb, when similarly treated.

This view was tested further by collecting the reaction mixture as it left the observation tube in a test tube half filled with CO-saturated buffer containing $Na_2S_2O_4$. When the test tube was full, the solution, now 1 in 120 with respect to the original blood, was examined immediately with a hand spectroscope or a Hartridge reversion spectroscope. The light path could be made to traverse up to 1·5 cm. of liquid. When O_2Hb (partial pressure of $O_2 = 0{\cdot}2$–$1{\cdot}0$ atm.) and buffered $Na_2S_2O_4$ were allowed to react and were examined in this way, a faint but definite band was visible at 628 mμ. in one experiment. No such band was observed when reduced haemoglobin and $Na_2S_2O_4$ were allowed to react and were similarly examined. The 'plateau' in this experiment was at about 18 % O_2Hb, i.e. in the higher range.

The blood used in this experiment was stored overnight in the refrigerator and the experiment repeated next day under as closely similar conditions as possible. In this case the band in the red was less pronounced, and the 'plateau' was found to have dropped to about 3 % O_2Hb. As to the cause of the difference of behaviour on the two successive days we have at present no clue, but the correlation of the pronounced 'plateau' with the appearance of the band in the red is striking. The conditions under which the latter was obtained are consistent with the supposition that CO-choleglobin (Legge & Lemberg, 1941) or perhaps CO-cruoralbin (Holden, 1943) had appeared.

For the present purpose it is unimportant whether choleglobin or cruoralbin is formed. Although the band observed was that of a ferrous CO compound (the CO being used to stop further coupled oxidation and to form a derivative with a sharp banded spectrum), the ferrous forms of both the above compounds have absorption bands in the 620–630 mμ. region in the absence of CO. The presence of either, in sufficient amount, could invalidate our interpretation of the galvanometer deflexion in terms of only oxy- and reduced haemoglobin. In view of the arbitrary way in which the galvanometer was adjusted to zero by movement of the red-green filter it is not possible to state, on the basis of the published data on the absorption of choleglobin or cruoralbin, what concentration of these compounds would be required in order to simulate a given oxyhaemoglobin-reduced haemoglobin mixture in the apparatus.

Additional evidence that part of the 'diphasic' course of the reduction may be accounted for by the presence of other pigments is provided by a few experiments which were carried out for us by Dr B. Chance with his refined photoelectronic rapid reaction apparatus. In these experiments monochromatic light of wavelength 578 mμ. was used, the percentage reduction being obtained by the difference in absorption of oxy- and reduced haemoglobin at this wavelength. The reduction appeared to be a first order reaction all the way, no significant second phase being detected.

This tentative explanation of the 'diphasic' reduction of oxyhaemoglobin does not, however, account for all the aspects of the phenomenon. The fact that a slow decline in the apparent concentration of oxyhaemoglobin is observed during the second phase of the reduction, is inconsistent with the supposition that the concentration of choleglobins is increasing at this period in the course of the reactions, and suggests that a fourth compound, in addition to oxyhaemoglobin, reduced haemoglobin and choleglobin (or cruoralbin), may be present. This might be a precursor of the choleglobin observed spectroscopically 2–3 sec. after mixing. Further investigation of the problem, however, will have to await an apparatus such as the one constructed by Chance (1947), which is able to record the absorption at several wavelengths simultaneously.

Although the matter has not yet been fully worked out, we do feel it reasonable to conclude that a large part of the apparent 'plateau' when the latter is high, is due to the appearance of abnormal pigments, rather than to a genuine diphasic dissociation of oxyhaemoglobin. We cannot, however, at present exclude the possibility that the latter may exist in a minor degree, but to be sure of this it would be most desirable to use gasometric as well as spectroscopic methods of detection. If, and only if, such combined tests turned out positive, would it be worth while to consider further the bearing of the 'plateau' upon the three questions, listed as (i), (ii), and (iii) at the beginning of the present section 2C. As regards the apparently faster initial rate of dissociation of the partially saturated haemoglobin, shown in Fig. 7, it may be largely due to the presence of a smaller proportion of oxidized $Na_2S_2O_4$ products than in the case of the fully saturated haemoglobin.

Two other possible explanations of the 'plateau' we believe have already been ruled out. (i) Hydrogen peroxide is known to be formed during the oxidation of $Na_2S_2O_4$ and this might be decomposed by the blood catalase and the oxygen so formed reoxygenate the haemoglobin, thus setting up a temporary cyclic process. In a control experiment in which sodium azide was added to give a final concentration of 0·001 M-sodium azide (so as to inhibit completely the catalase of the blood solution) no difference in the diphasic course of the reaction was found, the plateau remaining at approx. 18 % O_2Hb. (ii) That the $Na_2S_2O_4$ may only react slowly with dissolved O_2, at the very low partial pressures of the latter which must exist during the last stages of dissociation of O_2Hb. If so, the red-cell suspensions should also be similarly affected, which they are not: furthermore, the speed of the slow phase should be dependent on the concentration of $Na_2S_2O_4$, which has already been shown not to be the case.

In conclusion it may be noted that dilute solutions of haemoglobin and of $Na_2S_2O_4$ are both much more labile at pH 7 and below (i.e. the range in which these effects appear) than at alkaline pH. Although $Na_2S_2O_4$ has proved of great service in studying the kinetics of dissociation of oxygen-carrying pigments, it is a great pity that no satisfactory alternative oxygen absorbent has been discovered for this purpose, particularly in the neutral pH range.

SUMMARY

1. The rate of combination of X with haemoglobin (Hb), where X = oxygen or carbon monoxide, has hitherto been expressed by the equation $d[XHb]/dt = k'[X][Hb] - k[XHb]$, which has also been applied to the dissociation of X from haemoglobin. In the later stages of combination, however, it is now found that the calculated value of k' tends to rise appreciably, whereas in the early stages of dissociation of oxyhaemoglobin (O_2Hb), k tends to be much lower than the plateau value which it attains after the dissociation is about one-third completed. These discrepancies are in line with expectations based on the intermediate compound theory.

2. In all previous kinetic work the haemoglobin at time zero has been either completely in the form XHb, or in the form Hb. Theoretically discrepancies might occur if the haemoglobin was partially saturated at the outset of the reaction. Experimental tests, however, gave negative results in the case of combination of carbon monoxide with Hb at pH 6·8 and 10·0, and also in the dissociation of O_2Hb in presence of $Na_2S_2O_4$ at pH 10·0, but at pH 6·8 a slight positive effect was observed with the latter reaction.

3. At pH 6·8, and at neighbouring pH, the dissociation of O_2Hb in presence of sodium dithionite ($Na_2S_2O_4$) seems to occur in two phases, a fast one from 100% O_2Hb to 20 ± 10% O_2Hb, followed by a slow final one taking several seconds. Control showed that a large part of this effect is probably due to secondary effects of the oxidation products of $Na_2S_2O_4$ upon Hb, leading to formation of choleglobin and related products, the presence of which might well interfere with the estimation of the O_2Hb/Hb ratios by the Millikan photocolorimetric method used in this paper.

4. New determinations are given of the comparative rates of combination of carbon monoxide with sheep haemoglobin in solution and in red-cell suspensions. Similar experiments on the rates of dissociation of O_2Hb in solution and in the red cell are also given.

The work described in this paper was carried out during the tenure by J. W. L. of a Research Fellowship from the Wellcome Trustees.

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Biochem. Journal (Ukraine)
16: 215-241 (1940)

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On the Effect of Oxidizing and Reducing Agents on the Hydrolytic Activity of the Pancreatic and Hepatic Lipases

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In the previous investigations the authors established that ascorbic acid can inhibit the hydrolytic activity of the pancreatic lipase up to a complete inactivation of this ferment. This inhibition is reinforced by the addition of even small amounts of copper sulfate and diminished under the influence of substances which inhibit the autooxidation of ascorbic acid. In order to study in more details the conditions influencing this process, the authors studied in the present paper the effect of ascorbic acid on the pancreatic and hepatic lipase and also the effect of some oxidizing and reducing agents on these ferments. The results of these investigations showed that the inhibition of pancreatic lipase by ascorbic acid is probably of irreversible nature, as under the following action of hydrogen sulfide or sodium hydrosulfite no restoration of the lost activity is observed. No spontaneous regeneration of the ferment activity is observed in experiments with prolonged (20—24 hours) exposition of ascorbic acid with pancreatic lipase. In the presence of iron salts (Fe^{++} and Fe^{+++}) the inhibition of the pancreatic lipase by ascorbic acid is lower as in adding copper sulfate, and even lower as the inhibition produced by the ascorbic acid itself. Ascorbic acid + Fe^{++} or Fe^{+++} did not affect considerably the hydrolytic activity of the hepatic

lipase (tributyrine). In contrast to the non-fermentative catalysis, the enzymic oxidation of ascorbic acid (ascorbinase of cucumber juice) has no inhibiting effect on the hydrolysis of tributyrine of pancreatic lipase. The absence of an inhibition of methyl-butyrate hydrolysis by hepatic and by pancreatic lipase under the influence of ascorbic acid is also of great interest.

The system cysteine ⇆ cystine has the same effect on the pancreatic and hepatic lipase as the system ascorbic ⇆ dehydro-ascorbic acid. Cysteine inhibits the pancreatic lipase, cystine has no effect altogether.

Experiments with the influence of some chemical oxidizing and reducing agents showed that the reducing agents do not inhibit the hydrolytic activity of lipase (H_2S, $NaHSO_3$ and $Na_2S_2O_4$); permanganate which oxidizes by atomary oxygen inhibits very actively, hydrogen superoxide has a very weak and irregular effect; ferricyanide and methylene blue have no influence on the activity of the ferment. The differences between the hepatic and pancreatic lipases in regard to their comportment with different oxidizing and reducing agents are more of a quantitative character (the hepatic lipase is somewhat more resistent to the effect of oxidizers than pancreatic lipase).

The results of these experiments disprove the supposition that the inhibiting effect of ascorbic acid and cysteine is conditioned by the hydrogen atoms which are split off during the process of dehydratation. The supposition is more probable that during the autooxidation of these products intermediate products with a higher oxidizing potential are formed, which include into the oxidation some enzyme groupings that are more resistent to molecular oxygen or accompanying substances that are acting as activators.

Finally, resuming the results obtained, the authors want to emphasize the following two moments:

1. In experiments in vitro the inhibiting effect connected to oxidation-reduction processes was established much more sharply than the weak and instabil, activating effect. The opposite effect of these processes on the hydrolytic and synthetic activity of pancreatic lipase is not a regular law [1]).

[1]) In collaboration with K. I. Strachitsky the authors have established an inhibiting action of ascorbic acid and cysteine on the synthetic activity of this lipase.

This proves more complex relationships between the reversibility of the action of pancreatic lipase and the oxidation-reduction processes. These relationships are not reflected by the simple scheme oxidation—synthesis, reduction—hydrolysis.

2. The differences established by the authors in the effect of fermentative and non-fermentative oxidation of ascorbic acid on pancreatic lipase put forward the problem on the influence of different systems taking part in the processes of hydrogen and oxygen transportation on the activity of lipolytic ferments.

In this respect further researches are carried out by the authors.

Biochem. J. (1957) 67(4): 572-579, 1957

The Enzymic Hydrolysis of Adenosine Triphosphate by Liver Mitochondria

2. EFFECT OF INHIBITORS AND ADDED COFACTORS

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(*Received* 4 *March* 1957)

In the previous paper (Myers & Slater, 1957*a*), experiments were reported which were interpreted on the basis that four different enzymes or enzyme systems were responsible for the hydrolysis of adenosine triphosphate (ATP) by liver mitochondria, under various conditions. Each of these systems appeared to be relatively specific for ATP. Three of the four enzyme systems were activated by dinitrophenol (DNP), which suggested that they contained components which might be involved in oxidative phosphorylation.

In the present paper, the effect of various inhibitors of the respiratory chain on the hydrolysis of ATP is examined. This investigation was undertaken in order to determine whether any of the members of the respiratory chain are directly involved in the enzymic hydrolysis of ATP by mitochondrial preparations, as has been suggested in some formulations of oxidative phosphorylation (e.g. Slater, 1953*a*).

A preliminary report of some of these results has appeared (Myers & Slater, 1957*b*).

METHODS

All the methods have been described in the previous paper (Myers & Slater, 1957*a*).

The diphosphopyridine nucleotide (DPN) was obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A., and the reduced diphosphopyridine nucleotide (DPNH) was prepared by reduction with alcohol dehydrogenase, as described by Slater (1953*b*).

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RESULTS

Cyanide. Chance & Williams (1956) have shown that freshly prepared liver mitochondria contain endogenous substrate, which is rapidly exhausted by the addition of phosphate acceptor or an uncoupling agent such as DNP in the presence of oxygen. One would expect that under the conditions of the measurement of the ATPase activity in this paper, the endogenous substrate would be rapidly exhausted and that all the components of the respiratory chain would be completely oxidized for the greater part of the assay. So far as the cytochrome system was concerned, this was confirmed by direct spectroscopic observation. The addition of 10^{-3} M-KCN prevented the oxidation of endogenous substrate, and maintained the cytochromes (and presumably also the pyridine nucleotides and flavoproteins) in the reduced state during the whole course of the assay. However, Fig. 1*A* shows that this addition had no effect on the ATPase activity throughout the range of pH values studied.

Similarly, the addition to the Keilin & Hartree (1947) heart-muscle preparation of 10^{-3} M-KCN, either alone or in the presence of 0·01 M-succinate (added to reduce the respiratory chain, since this preparation is free from endogenous substrate), had no effect on the ATPase activity. It must be concluded therefore that neither cytochrome a_3, which combines with cyanide, nor the other members of the respiratory chain which undergo oxidation and reduction under these conditions are likely to be involved in the hydrolysis of ATP by mitochondrial preparations.

Inhibitors of succinic dehydrogenase. The participation of succinic dehydrogenase in the hydrolysis of ATP by the Keilin & Hartree heart-muscle preparation was excluded by incubation with 0·02 M-KCN for 3 hr. at 38° and pH 7·4. Under these conditions, succinic dehydrogenase is completely inactivated (Tsou, 1951). Nevertheless, there was no effect on the rate of hydrolysis of ATP at any pH value between 5 and 10. Further evidence that the succinic dehydrogenase is not involved in this reaction was afforded by lack of effect of 0·01 M-malonate on the ATPase activities of preparations of liver mitochondria.

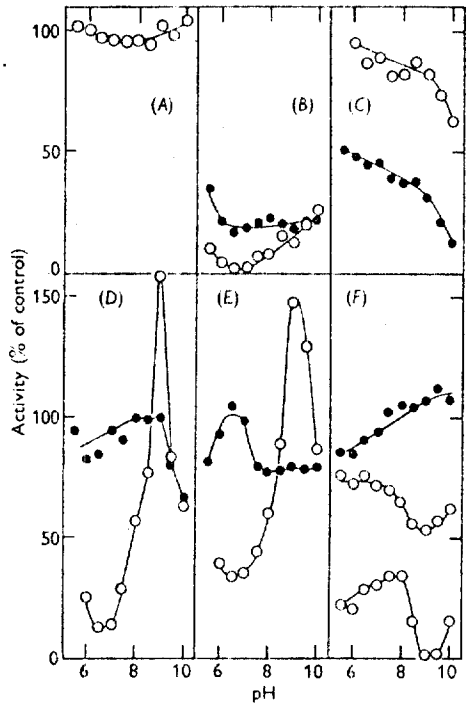

Fig. 1. Effect of various inhibitors on the hydrolysis of ATP by liver mitochondria. Data are presented for the ATPase activities of normal mitochondria in the presence of 10^{-4} M-DNP (○) and of aged and frozen mitochondria (Myers & Slater, 1957*a*) (●); a significant stimulation of the ATP hydrolysis by normal mitochondria in the presence of 10^{-4} M-DNP is produced only between pH 6 and 8·5 (see Myers & Slater, 1957*a*). The following inhibitors were used: (*A*) 10^{-3} M-KCN; (*B*) 10^{-3} M-NaN_3; (*C*) 0·01 M-guanidine hydrochloride; (*D*) 10^{-4} M-*p*-chloromercuribenzoate; (*E*) 0·06 M-KF; (*F*) bovine plasma albumin in concentrations of 5, 3·3 and 10 mg./ml. from top to bottom. The reaction mixture with the normal mitochondria was: KCl (0·075 M); sucrose (0·05 M); tris buffer (0·05 M); $MgCl_2$ (0·001 M); ethylenediaminetetra-acetic acid (6×10^{-4} M); ATP (0·002 M). With aged mitochondria the ethylenediaminetetra-acetic acid was omitted.

Antimycin and 2:3-dimercaptopropanol. Both of these compounds can interrupt the respiratory chain at some point between cytochrome *b* and cytochrome *c* (Slater, 1949; Potter & Reif, 1952). A concentration of antimycin sufficient to stop respiration (10^{-6} M) had no significant effect on the hydrolysis of ATP by normal mitochondria in the presence of DNP or by aged and frozen mitochondria between pH 5 and 10. Higher concentrations of antimycin stimulated the hydrolysis of ATP by normal mitochondria in the absence of DNP. Incubation of the Keilin & Hartree heart-muscle preparation with 0·01 M-2:3-dimercaptopropanol for 30 min. at 37°, a procedure sufficient to inactivate the respiratory chain completely (Slater, 1949), caused only a slight inactivation of the ATPase at all pH values.

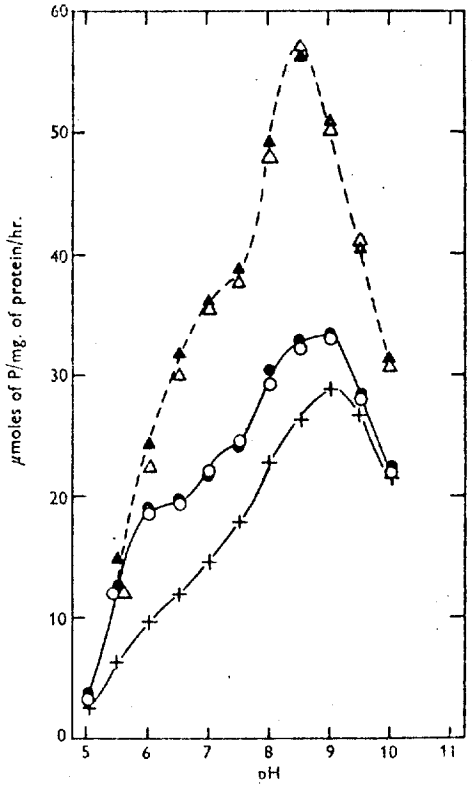

Fig. 2. Hydrolysis of ATP by liver mitochondria after extraction of the cytochrome *c*, as described by Schneider *et al.* (1948). Data are given for the extracted mitochondria in the absence of DNP (○), in the presence of 10^{-4} M-DNP (△) and in the presence of 10^{-4} M-DNP + 10^{-4} M-cytochrome *c* (●). The same mitochondria were also assayed in the absence (△) and the presence (▲) of 10^{-4} M-cytochrome *c* after the mitochondrial preparation had been aged and frozen. The reaction mixtures were the same as in Fig. 1.

Addition and removal of cytochrome c. The hydrolysis of ATP by mitochondria in the presence or absence of DNP was scarcely affected by the addition of 7×10^{-5}M cytochrome *c*, even after the mitochondria were washed twice with water at 0° and subsequently twice with 0·15M-NaCl at 0° in order to extract the cytochrome *c* (Schneider, Claude & Hogeboom, 1948). Although the exposure to hypotonic conditions activated the latent ATPase activity to a considerable extent, the addition of DNP still stimulated the ATPase activity (Fig. 2). After this mitochondrial preparation was aged and frozen (Myers & Slater, 1957*a*), the pH activity curve of the fully activated ATPases appeared to be the same as obtained with a similar preparation from normal mitochondria [cf. Fig. 2 with Fig. 8 of the previous paper (Myers & Slater, 1957*a*)]. The curve was not affected by the addition of 10^{-4}M-cytochrome *c*, either alone or in the presence of dithionite ($Na_2S_2O_4$) in order to reduce the cytochrome *c*. The effect of dithionite itself on the ATPase activity will be described below.

Removal and addition of diphosphopyridine nucleotide and reduced diphosphopyridine nucleotide. Both aged liver mitochondria and the Keilin & Hartree heart-muscle preparation, which contain little DPN, exhibit a high ATPase activity, which was not affected by the addition of 10^{-3}M-DPN or DPNH. The same concentration of DPNH also had no effect on the ATPase activity of fresh liver mitochondria in the presence of DNP, between pH 5·5 and 8·5.

α-Tocopherol and vitamin K_1. There are some indications that these two vitamins may also be involved in oxidative phosphorylation, either as members of the respiratory chain or as essential cofactors (Martius, 1956; Nason & Lehmann, 1956; Bouman & Slater, 1956). The Keilin & Hartree heart-muscle preparation contains an appreciable amount of tocopherol, but no vitamin K (Bouman & Slater, 1956). The ATPase activities of the Keilin & Hartree heart-muscle preparation were not affected by the addition of 0·07 mg. of DL-α-tocopherol or 0·02 mg. of vitamin K_1/ml. of reaction mixture; the stable stock suspensions used in these experiments were prepared in sucrose solutions containing 10% of ethanol and 0·5% of plasma albumin (cf. Nason & Lehmann, 1956). The same amount of tocopherol also had no effect on the ATPase activities of normal liver mitochondria in the presence of DNP.

Azide. One of the most potent inhibitors of the ATPase activities of mitochondrial preparations is sodium azide (Meyerhof & Ohlmeyer, 1952; Novikoff, Hecht, Podber & Ryan, 1952; Sacktor, 1953; Robertson & Boyer, 1955; Maruyama, 1954, 1956). However, its action is rather complicated since it also uncouples oxidative phosphorylation (Loomis & Lipmann, 1949) and can stimulate as well as inhibit the ATPase activity of normal liver mitochondria (Robertson & Boyer, 1955; Swanson, 1956). Our experiments showed that the stimulation of the ATPase activity by azide in the absence of DNP precedes the slower inhibitory reaction; the major portion of the ATP hydrolysis occurred within the first 5 min. after adding the normal liver mitochondria to a reaction mixture containing 10^{-3}M-NaN_3. The slow rate of the inhibitory reaction could also be demonstrated by experiments in which the mitochondria were incubated with azide before the ATP was added (Fig. 3); the stimulation by the azide became much less after pre-incubation for 10 min. and disappeared completely when the mitochondria were pre-incubated for 20 min. The stimulation of the latent ATPase activity of normal mitochondria by 10^{-3}M-NaN_3 was maximal at about pH 6 (Fig. 4); this result is similar to that observed with low concentrations of DNP (Myers & Slater, 1957*a*).

The same concentration of azide produced a strong inhibition of the ATPase, activated by the addition of DNP to normal mitochondria or by ageing and freezing the mitochondrial suspensions (Fig. 1*B*). A similar inhibition was observed with heart sarcosomes and with the Keilin & Hartree heart-muscle preparation.

Calcium. Low concentrations of Ca^{2+} ions cause a reversible uncoupling of oxidative phosphorylation, and higher concentrations result in a number of irreversible effects (Potter, Siekevitz & Simonson, 1953; Chance & Williams, 1956). As would be

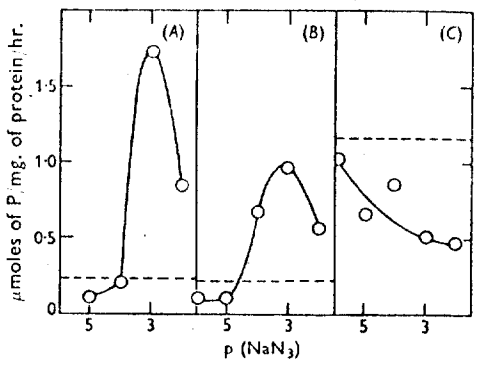

Fig. 3. Effect of sodium azide on the hydrolysis of ATP by normal liver mitochondria. The initial reaction mixture contained 0·075M-KCl, 0·108M-sucrose, 0·001M-ethylenediaminetetra-acetic acid, 0·003M-$MgCl_2$, pH 7·4. p (NaN_3) indicates the negative logarithm of the molar concentration of NaN_3 added. The mitochondria were pre-incubated in these reaction mixtures with or without azide for (*A*) 0, (*B*) 10 and (*C*) 20 min. before adding a small volume of ATP solution (final concn.: 0·002M-ATP). The control activities in the absence of azide are indicated by the horizontal broken lines.

expected, low concentrations of Ca^{2+} ions (10^{-3}M) also stimulated the latent ATPase activity of normal mitochondria (cf. Lardy & Wellman, 1953; Potter *et al.* 1953), which was maximal at about pH 6·5 (Fig. 4). The same concentration inhibited the ATPase activity of normal mitochondria at high pH values, and a similar inhibition above pH 9 was also observed with mitochondria, fully activated by ageing followed by freezing and thawing. This inhibition at high pH, which was also obtained with Mg^{2+} ions (Myers & Slater, 1957*a*), is probably due to combination with the ATP (Martell & Schwarzenbach, 1956).

p-*Chloromercuribenzoate*. This compound is a potent inhibitor of the mitochondrial ATPase at pH 7·4 (Novikoff *et al.* 1952; Sacktor, 1953; Lardy & Wellman, 1953; Hunter, 1956) but, like azide, can also activate the latent ATPase activity of normal mitochondria (Hunter, 1956). However, the activation occurred at all pH values from 5·5 to 9 (Fig. 4) and was not restricted to the lower pH values as was found with low concentrations of DNP, azide and Ca^{2+} ions. Alterations in the translucency of the normal mitochondrial suspensions were also observed (cf. Hunter, Davis & Corlat, 1956). The stimulation of the latent ATPase activity by *p*-chloromercuribenzoate may therefore be due to a general damage of the mitochondrial structure rather than to a specific uncoupling action.

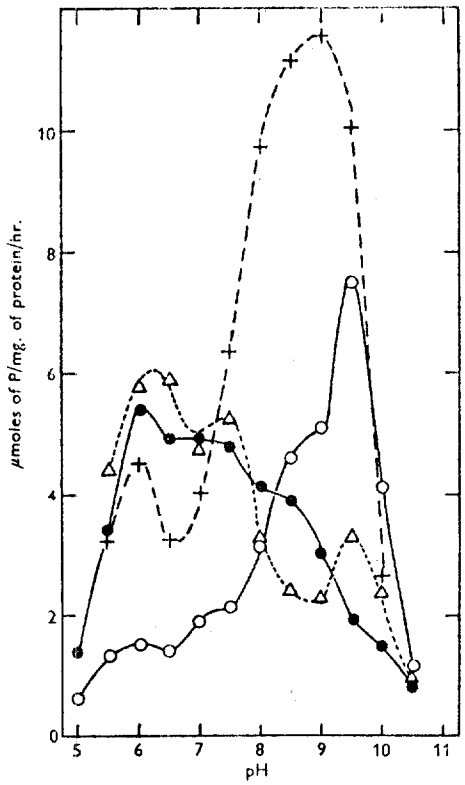

Fig. 4. Stimulation of the ATPase activities of normal liver mitochondria by various compounds. The reaction mixture contained 0·075M-KCl, 0·05M-sucrose, 0·05M-tris buffer, 0·002M-ATP, 0·001M-$MgCl_2$ and 0·0006M-ethylenediaminetetra-acetic acid. The ATPase activities were measured in the presence of 10^{-3}M-$CaCl_2$ (△), 10^{-3}M-NaN_3 (●) and 10^{-4}M-*p*-chloromercuribenzoate (+), the enzymic reaction being allowed to proceed for 15 min. in the first case and for 5 min. in the second and third cases. The control activities in the absence of these compounds were similar in these experiments and the average values obtained are shown above (○).

The degree of inhibition of the activated ATPase by *p*-chloromercuribenzoate was highly dependent on the Mg^{2+} ions and salt concentrations in the reaction medium and on the state of the mitochondria (Lardy & Wellman, 1953). The ATPase of normal mitochondria in the presence of DNP was strongly inhibited by 10^{-4}M-*p*-chloromercuribenzoate between pH 6 and 8, but a stimulation was observed in the region of pH 9 (Fig. 1*D*), where DNP has little effect on the latent ATPase activity but where the *p*-chloromercuribenzoate itself also stimulated in the absence of DNP (cf. Fig. 4). The same concentration of *p*-chloromercuribenzoate had little effect on the hydrolysis of ATP by mitochondria activated by ageing followed by freezing and thawing (Fig. 1*D*). A higher concentration (10^{-3}M) also had little effect. This might suggest that the hydrolysis of ATP by normal mitochondria in the presence of DNP follows a different pathway from the hydrolysis by aged and frozen mitochondria; however, the lack of inhibition by *p*-chloromercuribenzoate in the latter case could be due to the liberation of sulphydryl groups during the process of ageing, and a consequent protection of the ATPases against the inhibitory action. The ATPase activities of the Keilin & Hartree heart-muscle preparation were inhibited 40–60% by 10^{-3}M-*p*-chloromercuribenzoate.

Fluoride. This compound is a strong inhibitor of the pyrophosphatase and adenylkinase (Barkulis & Lehninger, 1951; Swanson, 1952; Sacktor, 1953; Siekevitz & Potter, 1953), but has less effect on the ATPase activity of liver mitochondria at pH 7·4 (Kielley & Kielley, 1951; Novikoff *et al.* 1952; Sacktor, 1953; Lardy & Wellman, 1953; Potter *et al.* 1953). However, the hydrolysis of ATP by normal mitochondria in the presence of DNP could be inhibited by high concentrations of KF (0·06M) between pH 6 and 8; as with *p*-chloromercuribenzoate, an activation was observed at higher pH values (Fig. 1*E*). On the other hand, 0·06M-KF had little effect on the hydrolysis of ATP by aged and frozen mitochondria (Fig. 1*E*), aged mitochondria or the Keilin & Hartree heart-muscle preparation

We did not observe either an uncoupling of oxidative phosphorylation (cf. Middlebrook & Szent-Györgyi, 1955) or a stimulation of the latent ATPase activity of normal mitochondria by high concentrations of KBr and KI. On the other hand, high concentrations of KF did exert some uncoupling action (cf. Kielley & Kielley, 1951) and also stimulated the latent ATPase activity of normal mitochondria in the absence of DNP. However, an appreciable stimulation was observed only in the absence of Mg^{2+} ions, possibly because the mitochondria are then more fragile. The concentration of fluoride (about 0·01 M) used by Copenhaver & Lardy (1952) and most other investigators in experiments on oxidative phosphorylation, had no significant effect on the latent ATPase activity of normal mitochondria in the presence of 0·001 M-$MgCl_2$ and 0·0006 M-ethylenediaminetetra-acetic acid.

Guanidine. Hollunger (1955) has shown that guanidine inhibits the hydrolysis of ATP by aged mitochondria at pH 7·4 but has little effect on the hydrolysis by normal mitochondria in the presence of DNP. These observations were confirmed over the whole pH range in the present investigation, with 0·01 M-guanidine (Fig. 1C). These results might also indicate different pathways for the ATPase activities in normal and aged preparations. However, the ATPase activity of normal mitochondria in the presence of DNP can be inhibited by higher concentrations of guanidine.

Albumin. The effect of plasma albumin was of particular interest because it is reported to combine with mitochrome, a haemoprotein which is liberated from aged mitochondria, and it uncouples oxidative phosphorylation and stimulates the latent ATPase activities of normal mitochondria in the same way as DNP; both effects are prevented by the addition of serum albumin (Shmukler & Polis, 1953; Polis & Shmukler, 1954).

No evidence could be obtained to suggest that mitochrome played a role in the activity of the ATPase of our aged and frozen suspensions of liver mitochondria. Bovine plasma albumin (fraction V from Armour Laboratories) in concentrations up to 10 mg./ml. (see Fig. 1) and ovalbumin (up to 50 mg./ml.) had no effect. Mitochondrial fragments obtained in such a way as to extract soluble proteins, by the procedure of Shmukler & Polis (1953), still possessed a high ATPase activity, which was not increased by adding the 'soluble' fraction (see Table 1). The high activity of the Keilin & Hartree preparation and of the mitochondrial fragments of Kielley & Kielley (1953) also speak against an easily soluble enzyme being concerned in these activities, since soluble enzymes are removed from these preparations.

Albumin had no effect on the stimulation of the ATPase by azide, but markedly decreased the activity in the presence of DNP (Fig. 1*F*). However, the significance of this apparent inhibition is uncertain since the inhibition was maximum at pH 9·4, where DNP no longer stimulates. Albumin does not prevent the uncoupling action of DNP (Slater & Lewis, 1954).

Dithionite. Dithionite caused a marked activation of the ATPase activity of aged and frozen mitochondria between pH 6 and 9 (Fig. 5). The degree of stimulation by 10^{-3} M-$Na_2S_2O_4$ was maximum at pH 7·5, and negligible above 9; it was not increased when the concentration of dithionite was increased to 10^{-2} M. The activation by 10^{-3} M-$Na_2S_2O_4$ was not affected by the addition of catalase, indicating that it was not caused by H_2O_2 produced by the auto-oxidation of $Na_2S_2O_4$. There was no stimulation of the ATPase activity of normal mitochondria in the presence of 10^{-4} M-DNP; however, this experiment is not conclusive since the $Na_2S_2O_4$ altered the colour of the DNP from yellow to orange.

Other reducing agents did not give the same effect as $Na_2S_2O_4$. Thus 10^{-3} M-$K_4Fe(CN)_6$ brought about only a slight activation, and 10^{-3} M-glutathione (cf. Novikoff *et al.* 1952) and 10^{-3} M-ascorbic acid were slightly inhibitory (Fig. 5). An oxidizing agent, 10^{-3} M-$K_3Fe(CN)_6$, also had no effect on the ATPase activities of aged and frozen mitochondria.

Table 1. *Enzymic hydrolysis of adenosine triphosphate by the soluble and insoluble fractions of aged liver mitochondria*

Normal liver mitochondria were suspended in 0·15 M-KCl + 0·02 M-$NaHCO_3$ and aged by incubation for 80 min. at 30°. The 'soluble' fraction was recovered by centrifuging off the mitochondria at 27 000 *g*. The insoluble mitochondrial material was washed with the salt solution, resuspended and incubated for a further 80 min. at 30° to complete the extraction of soluble proteins. The mixture was again centrifuged at 27 000 *g*, and the sediment washed and resuspended. This constituted the insoluble fraction. The reaction mixture for the ATPase assay contained 0·075 M-KCl, 0·05 M-sucrose, 0·05 M-tris buffer, 0·002 M-ATP and 0·001 M-$MgCl_2$.

pH	ATPase activity (μmoles of P/mg. of protein/hr.)		ATPase activity of the soluble and insoluble fractions combined (calc. as % of the sum of their separate activities)
	Insoluble fraction	'Soluble' fraction	
5·5	10·7	2·9	94
6·0	15·9	2·6	93
6·5	18·7	2·6	95
7·0	22·3	2·8	92
7·5	25·2	3·0	90
8·0	30·1	3·7	86
8·5	34·0	4·1	81
9·0	32·1	3·7	83
9·5	27·8	2·7	95
10·0	22·0	1·7	96

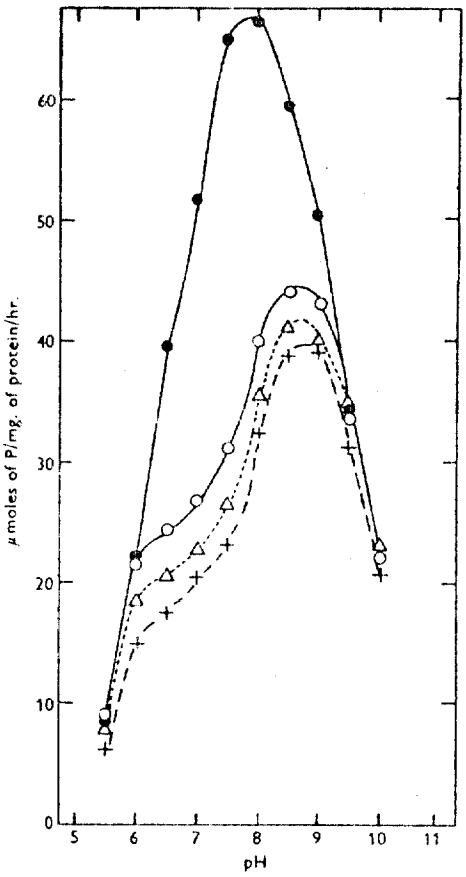

Fig. 5. Effect of reducing agents on the ATPase activities of aged and frozen liver mitochondria. The reaction mixture is the same as that given in Table 1. ATPase activities were measured in the absence of further addition (○) and in the presence of 10^{-3}M-sodium dithionite (●), 10^{-3}M-glutathione (△) and 10^{-3}M-ascorbic acid (+).

DISCUSSION

The coupling of phosphorylation with the transfer of hydrogen (or electrons) through the respiratory chain has been formulated in its simplest terms by the reactions (1) and (2):

$$AH_2 + B + C \rightleftharpoons A \sim C + BH_2 \qquad (1)$$

$$A \sim C + ADP + H_3PO_4 \rightleftharpoons A + C + ATP \qquad (2)$$

where AH_2 and B are adjacent members of the respiratory chain, whose interaction is coupled with phosphorylation, and C is an intermediate linking the oxidation–reduction with the phosphorylation reaction (Slater, 1953*a*). It was postulated that DNP was in some way able to bring about the hydrolysis of A ~ C [reaction (3)]:

$$A \sim C + H_2O \xrightarrow{(DNP)} A + C. \qquad (3)$$

The hydrolysis of ATP stimulated by DNP is brought about by the reverse of reaction (2), followed by reaction (3).

According to this formulation, a member of the respiratory chain (A), as well as a component (C), which does not undergo oxidation–reduction, is directly involved in the hydrolysis of ATP. Therefore combination of A with an inhibitor or its reduction to AH_2 would be expected to inhibit the ATPase activity. In this paper it has been shown that the addition of inhibitors of the respiratory chain (cyanide, antimycin, malonate), or the removal or inactivation of certain components (DPN, cytochrome *c*, the 2:3-dimercaptopropanol-labile factor), or the reduction of all components of the respiratory chain by the addition of cyanide and substrate, did not cause any inhibition of the ATPase activity. Thus the minimum hypothesis represented by equations (1), (2) and (3) is no longer adequate, and must be modified to take A out of the 'ATPase' reaction. The simplest extension of the theory is to introduce an additional step, with an additional component, between reactions (1) and (2). Chance & Williams (1956) have also given kinetic reasons for introducing an additional component (X) between reactions (1) and (2). They have also pointed out that C in the above scheme has the properties of an inhibitor, since A ~ C is an inhibited form of A which cannot be reduced in the respiratory chain to AH_2. For this reason, Chance & Williams have replaced C by I (inhibitor). Since it is clearly desirable to avoid a multiplicity of symbols, we shall adopt the symbols X and I of Chance & Williams. We shall not, however, replace AH_2 and B by specific members of the respiratory chain, because we do not think that these have been definitely identified. The modified hypothesis then becomes:

$$AH_2 + B + I \rightleftharpoons A \sim I + BH_2 \qquad (1)$$

$$A \sim I + X \rightleftharpoons X \sim I + A \qquad (2)$$

$$X \sim I + ADP + H_3PO_4 \rightleftharpoons X + I + ATP \qquad (3)$$

$$X \sim I + H_2O \xrightarrow{(DNP)} X + I \qquad (4)$$

The hydrolysis of ATP is now explained by the reverse of reaction (3), followed by reaction (4). The latter reaction might involve DNP–I as an intermediate (Chance & Williams, 1956).

It is probable that there are three oxidation–reduction steps in the respiratory chain which are coupled with phosphorylation. It seems likely that each of the oxidation–reduction pairs (AH_2 and B)

involved would require a different mediator I. Thus there would be three different inhibitors which could bring about the hydrolysis of ATP in the presence of DNP by the reversal of reaction (3), followed by reaction (4). It is possible that the three different DNP-sensitive enzyme systems suggested in the previous paper (Myers & Slater, 1957*a*) are reactions involving the three different inhibitors. In the absence of evidence to the contrary, it is simplest to assume that X is the same in all cases and could, in fact, represent a general enzyme or coenzyme of respiratory-chain phosphorylation.

Assuming that the three peaks obtained under different conditions with DNP represent three different enzymes, all three are inhibited by azide, which suggests that they possess a common component which could be X. A sensitivity to azide naturally raises the possibility that a haematin compound is involved, although against this it should be noted that the inhibition was a slow reaction in contrast with the rapid reaction usually occurring between haematin compounds and azide. It is possible that X is mitochrome (Polis & Shmukler, 1954), but all attempts to demonstrate its action in our preparations failed. However, we must await further details of the preparation and properties of mitochrome before we can settle this point.

The ATPase activity at pH 9·4 is also inhibited by azide, although it is not stimulated by DNP. One possibility is that this enzyme involves X, but not I. It would be expected that reaction (3) of the new scheme would occur in two steps, e.g.:

$$X \sim I + H_3PO_4 \rightleftharpoons X \sim P + I \qquad (3a)$$

$$X \sim P + ADP \rightleftharpoons X + ATP \qquad (3b)$$

Some reaction of this type appears to be necessary to explain the rapid exchange between the oxygen atoms of inorganic phosphate and water catalysed by liver mitochondria (Cohn, 1953; Cohn & Drysdale, 1955; Boyer, Falcone & Harrison, 1954; Robertson & Boyer, 1955; Luchsinger, Falcone & Reynard, 1955). The hydrolysis of ATP and the lack of any effect of DNP would be explained if, at pH 9·4, $X \sim P$ reacts with water much more rapidly than with any of the inhibitors.

The mechanism of the stimulation of the latent ATPase activity by ageing and freezing the mitochondria is not clear, but the pH–activity curves suggest that the same enzymes (and therefore the same inhibitors) are involved. It seems likely then that hydrolysis of $X \sim I$ also occurs with these preparations.

SUMMARY

1. The addition to liver mitochondria of inhibitors of the respiratory chain (cyanide, antimycin, malonate), or the removal or inactivation of certain components (diphosphopyridine nucleotide, cytochrome *c*, the 2:3-dimercaptopropanol-labile factor), or the reduction of all components of the respiratory chain by the addition of cyanide and substrate, did not cause any inhibition of the adenosine triphosphatase activity. It appears very unlikely then that a member of the respiratory chain is directly involved in the hydrolysis of adenosine triphosphate (ATP).

2. When azide was added to liver mitochondria, the rate of hydrolysis of ATP was first stimulated (particularly at pH 6) and then inhibited. In the presence of dinitrophenol, the hydrolysis of ATP was inhibited over the whole pH range studied (5·5–10).

3. Calcium stimulated at acid pH values (maximum at pH 6·5) and inhibited at higher pH values.

4. *p*-Chloromercuribenzoate activated the ATPase at all pH values between 5·5 and 9, probably because of a general damage of the mitochondrial structure. In the presence of dinitrophenol the mercurial inhibited between pH 6 and 8 but stimulated at pH 9.

5. Fluoride has little effect on the ATPase activity of aged and frozen mitochondria, but in the presence of dinitrophenol inhibited the activity of normal mitochondria between pH 6 and 8, and stimulated at pH 9.

6. Guanidine, on the other hand, inhibited the systems in aged and frozen mitochondria more than those in normal mitochondria activated by dinitrophenol.

7. Albumin had no effect on the ATPase activity of aged and frozen mitochondria, or on mitochondria activated by azide, but markedly inhibited the activity in the presence of dinitrophenol, especially at alkaline pH values.

8. Dithionite strongly activated the ATPase of aged and frozen mitochondria, with a maximum at pH 7·5.

We wish to thank Mrs M. Jong-Tuynman for her skilled assistance in these experiments.

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Mikrobiologiya
No. 5, 1960
pp. 679-682

EFFECT OF SOME RADIOPROTECTORS ON THE MUTATION RATE OF THE UNSTABLE STRAIN OF BACTERIUM PRODIGIOSUM

A. A. Prozorov

Irradiation and chemical mutagenic factors are known to produce largely similar genetic effects. Besides acting directly on unique cell structures, irradiation forms compounds that possess mutagenic activity. Some investigators assume that natural, "spontaneous" mutagenesis is also caused by the radiomimetic properties of certain metabolites (Wagner and Mitchell, 1958). Radioprotective substances have been shown to prevent hereditary changes among other consequences of irradiation (Riley, 1955, 1957). There is no information on mutations whose appearance is not stimulated artificially. We therefore thought it worthwhile to study the effect of some radioprotective substances on natural mutagenesis.

In most organisms, it is difficult to reduce the rate of mutagenesis; the rate of spontaneous mutation is already low, except in the fairly rare cases of so-called unstable heredity when the frequency of mutation of any character increases greatly (Ryzhkov, 1939). This category apparently includes bacterial dissociation, particularly the dissociation of pigment formation in Bacterium prodigiosum (Labrum and Bunting, 1953; Kaplan, 1948, 1952, 1953).

We used this microorganism as a model to study the effect of radioprotective agents on natural mutation.

Procedure

We obtained a stock strain (B_1) of Bacterium prodigiosum from the Microbiology Department of the First Moscow Medical Institute. Grown on agar with 1% glucose, the strain produced colonies consisting of lighter and darker sectors (Figs. 1 and 2). In cultures taken with

a needle from any sector, two types of colonies inevitably grew: dark ones with one or two narrow light sectors and light ones with several wide dark sectors. The quantitative correlation between both types of colony transferred from the light and dark sectors was fairly constant when grown under the same conditions.

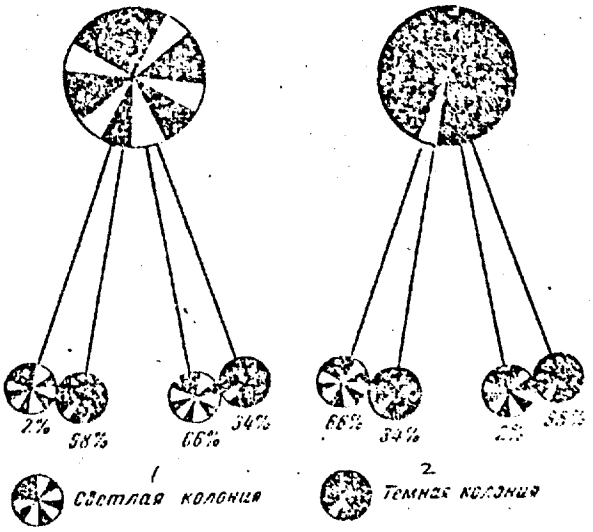

Fig. 1. Results of culturing different portions of the light and dark colonies of the unstable B_1 strain of Bacterium prodigiosum

1 - Light colonies
2 - Dark colonies

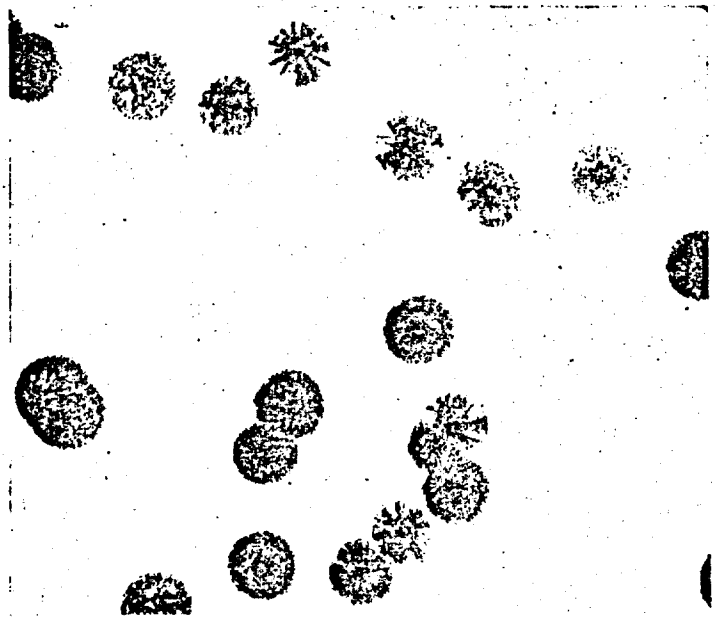

Fig. 2. Light and dark colonies of the unstable B_1 strain of Bacterium prodigiosum

Because the colonies were invariably heterogeneous in composition and this heterogeneity persisted after many passages (we passaged the B_1 strain for 2 years), it was reasonable to conclude that the formation of sectoral colonies was not a mechanical artifact (the combined growth of "adhering" "dark" and "light" cells) or a splitting in the offspring of a hybrid cell. The frequency with which "dark" cells appeared in a "light" population was much higher than the frequency of the reverse changes. Hence the greater width of the dark sectors and their greater number compared with the light sectors in the dark colonies.

We tried to alter the rate of mutation of "light" cells into "dark" ones, using the method of counting sectors of different areas proposed by Kaplan (1952) and Witkin (1951) to assess the results. We assumed that a mutation took place in the 2-cell stage in a colony consisting of 2"halves", light and dark, and in the 4-cell stage in a colony having a dark sector occupying approximately one-fourth the area, etc. Thus, by counting the number of sectors of each kind occurring on the average in a light colony we could compute the frequency of changes per generation in a

cell by dividing the result by the apparent number of cells corresponding to the formation of sectors of a given diameter in a colony. Even if the number found is different from the true frequency of mutation, it still can serve as a criterion when studying the effect of various substances on the rate of mutagenesis.

To study the effect of radioprotective substances on the mutation rate of Bacterium prodigiosum, we added them to melted agar and later counted the sectors in the grown colonies. Since some of the substances were toxic, we determined experimentally the minimum toxic concentration (under the given conditions) and then used subtoxic dilutions. The presence of antimutagenic action was checked in sodium hydrosulfite, cysteine, unithiol (a Soviet preparation similar in composition to the imported 2,3-dimercaptopropanol, the preparation BAL), and thiourea. We also tested the "known" mutagen potassium permanganate.

We used a binocular lens to count the mutation rate per generation. We took into account sectors occupying 1/2, 1/4, 1/8, and in some experiments 1/16 of the area of the colonies. Sectors were counted in isolated colonies at least 3 to 5 mm in diameter. The result of counting sectors in 25 to 30 colonies growing on a single dish was adopted as the statistical unit.

The data obtained in less than 30 experiments were treated in accordance with the formulas for small samples. The significance of the differences was judged with a probability factor of 0.95.

Results

Computation of the mutation rate by the described method yielded the values found in the "control" column in the table below. The frequencies of mutagenesis per generation counted for each class of sectors coincided fairly closely (except the sectors occupying half the area of a colony).

The reason why several large figures were obtained in the last case may have been that the "half" colonies also included colonies grown not from one but two cells, "light" and "dark", adhering together at the time of inoculation.

1 Размер сектора	2 Частота мутирования за генерацию			
	3 контроль	4 гидросульфит натрия (0,05%)	5 тиомочевина (0,025%)	6 перманганат калия (0,005%)
1/2	$10,0 \pm 1,6$ $\sigma = 3,2$ $n = 20$	$0,8 \pm 0,35$ $\sigma = 1,1$ $n = 36$	$7,0 \pm 1,3$ $\sigma = 3,4$ $n = 27$	$15,2 \pm 1,8$ $\sigma = 5$ $n = 30$
1/4	$7,6 \pm 0,8$ $\sigma = 1,6$ $n = 20$	$0,7 \pm 0,23$ $\sigma = 0,7$ $n = 36$	$3,9 \pm 0,57$ $\sigma = 1,4$ $n = 27$	$9,5 \pm 1,1$ $\sigma = 2,9$ $n = 30$
1/8	$6,7 \pm 0,8$ $\sigma = 1,7$ $n = 20$	$0,7 \pm 0,13$ $\sigma = 0,45$ $n = 36$	$4,0 \pm 0,57$ $\sigma = 1,4$ $n = 27$	$8,4 \pm 0,8$ $\sigma = 2,1$ $n = 30$
1/16	$6,7 \pm 0,06$ $\sigma = 0,1$ $n = 20$	—	—	—

Effect of Sodium Hydrosulfite, Thiourea, and Potassium Permanganate of the Mutation Rate of the B_1 Strain of Bacterium prodigiosum

1 - Size of sector
2 - Rate of mutation per generation
3 - control
4 - sodium hydrosulfite (0.05%)
5 - thiourea (0.025%)
6 - potassium permanganate (0.005%)

The effects of the radioprotective agents on the mutation rate are shown in the same table. Sodium hydrosulfite and thiourea slowed natural mutagenesis, the former more than 10-fold. The light colonies growing on agar with sodium hydrosulfite had only very narrow dark sectors which appeared to be continuous (Fig. 3).

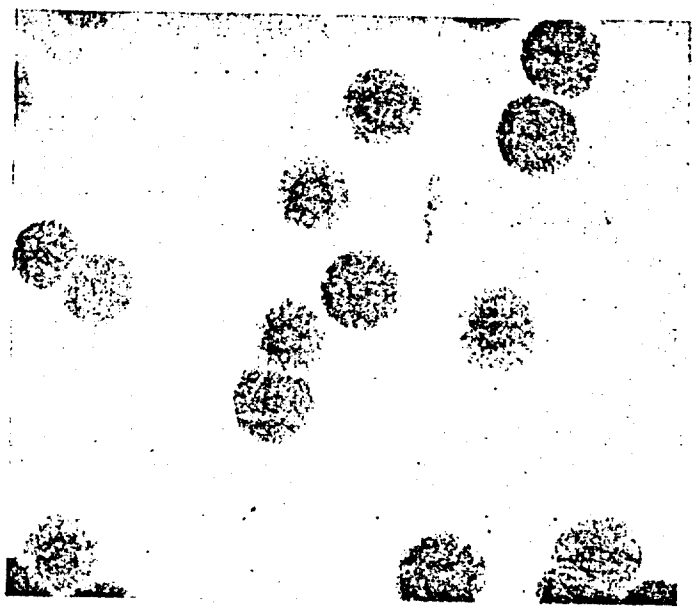

Fig. 3. Light and dark colonies of the unstable B_1 strain of Bacterium prodigiosum on sugar agar with 0.05% sodium hydrosulfite

Since no statistically significant differences were found between the mutation rate in the control and in the presence of unithiol (0.5%) or cysteine (0.01%), these data were not included in the table. Potassium permanganate accelerated mutagenesis.

The results of the experiments can be formally explained both by a slowing or acceleration of mutagenesis and by partial selection of "light" or dark" cells. However, if there had been partial inhibition of growth or some dieing off of a certain kind of cell ("dark"), there would have

been much fewer dark than light colonies on the agar with sodium hydrosulfite. However, the number of all the colonies was the same and only the area of the sectors decreased. Consequently, we were dealing with a true slowing of mutagenesis.

Discussion

The results of our experiments do not suggest that there is necessarily a connection between the radioprotective action of a substance and the antimutagenic effect. Nevertheless, the fact that substances with antimutagenic affect have been found is in itself a matter of interest. Very little information is available on the inhibition of natural mutagenesis by chemical agents, e.g., the work of Novick and Szillard (1952) who used adenosine to slow mutagenesis in E. coli. The presence of antimutagenic action (against natural mutagenesis) in some radioprotective agents has theoretical and possibly practical implications. Up to now geneticists concentrated on methods for increasing natural mutation in order to obtain new useful characters. However, it would have been preferable in some instances to slow such mutation (e.g., bacterial dissociation under industrial conditions).

Conclusions

1. A study was made of the mutation rate in the unstable strain of Bacterium prodigiosum without artificial treatment and with treatment by chemical agents.

2. Certain radioprotectors (sodium hydrosulfite, thiourea) slowed the rate of natural mutagenesis, whereas unithiol and cysteine did not have this effect. One of the mutagens (potassium permanganate) accelerated the mutation rate.

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Mikrobiologie; 29:679-682 (1960)

МИКРОБИОЛОГИЯ

Т. XXIX 1960 Вып. 5

ДЕЙСТВИЕ НЕКОТОРЫХ РАДИОЗАЩИТНЫХ ВЕЩЕСТВ НА СКОРОСТЬ МУТИРОВАНИЯ НЕСТАБИЛЬНОГО ШТАММА BACTERIUM PRODIGIOSUM

Л. А. Прозоров

Как известно, облучение клетки и воздействие химических мутагенных факторов дают во многом сходный генетический эффект.

По-видимому, помимо прямого действия на уникальные клеточные структуры, в результате облучения образуются соединения, обладающие мутагенной активностью. Имеются предположения, что естественный, «спонтанный» мутагенез также обусловлен радиомиметическими свойствами некоторых метаболитов (Вагнер, Митчелл, 1955). Известно, что радиозащитные вещества в числе других последствий облучения, предупреждают и образование наследственных изменений (Рили, Riley, 1955, 1957). Относительно других, воспроизводство которых искусственно не стимулируется, таких сведений нет. Поэтому мы считали интересным изучить влияние некоторых радиозащитных веществ на естественный мутагенез.

У бесчисленных организмов попытки уменьшить скорость мутагенеза затруднены и без того малой скоростью спонтанного мутирования. Исключение составляют довольно редкие случаи так называемой нестабильной наследственности, когда частота мутирования какого-нибудь признака возрастает во много раз (Рыжков, 1939). Видимо, к этой же категории можно отнести некоторые случаи диссоциации бактерий, в частности диссоциацию пигментообразования у Bact. prodigiosum (Лабрум и Бонснер, Labrum a. Bonsnes, 1948, 1952, 1953; Каплан, Kaplan, 1948, 1952, 1953).

Мы воспользовались этим микроорганизмом как моделью для изучения влияния радиозащитных веществ на естественную изменчивость.

Методика

Нами был взят музейный штамм $Б_1$ Bact. prodigiosum кафедры микробиологии 1-го Московского медицинского института, дающий на агаре с 1% глюкозы колонии, состоящие из секторов более светлого и более темного цвета (рис. 1 и 2). В рассевах микробной массы, взятой иглой из любого сектора, неизменно вырастали колонии двух типов: темные, с одним или двумя узкими светлыми секторами и светлые, с несколькими широкими темными секторами. Количественное соотношение между колониями обоих типов, высеваемых из светлых и темных секторов, при одинаковых условиях рассева было довольно постоянным (рис. 1).

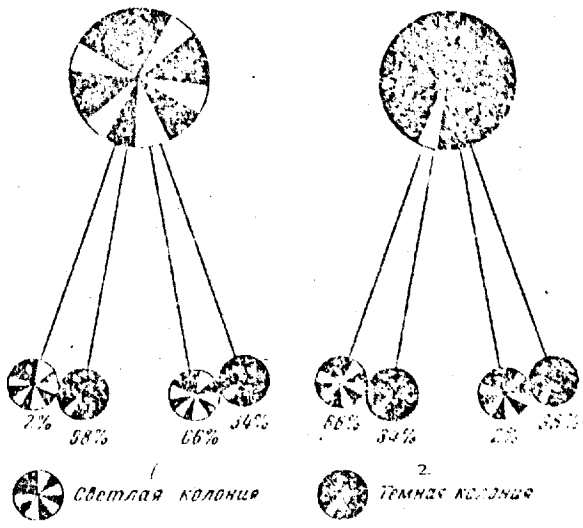

Рис. 1. Результаты рассева различных участков светлых и темных колоний нестабильного штамма $Б_1$ Bact. prodigiosum

Так как любая колония всегда была гетерогенной по составу и эта гетерогенность сохранялась после многочисленных пересевов (мы пассировали штамм $Б_1$ в течение двух лет), можно было заключить, что

образование секторных колоний нельзя считать механическим артефактом (совместным ростом «слипшихся» «темной» и «светлой» клеток) или расщеплением в потомстве гибридной клетки. Видимо, секторы в колониях образовывались за счет изменчивости клеток. При этом частота появления «темных» клеток в «светлой» популяции была значительно выше, чем частота обратных изменений. Отсюда — бóльшая ширина темных секторов и их большее количество по сравнению со светлыми секторами в темных колониях.

Мы старались изменить скорость мутирования «светлых» клеток в «темные». Для количественного учета был использован метод подсчета секторов различной площади, предложенный Капланом (1952) и Юткин (Witkin, 1951). Предполагалось, что в колонии, состоящей из двух «половинок» — темной и светлой,— мутация произошла на стадии двух клеток, в колонии, имеющей темный сектор, занимающий приблизительно четвертую часть ее площади — на стадии четырех клеток и т. д. Таким образом, подсчитав количество секторов каждого рода, приходящихся в среднем на одну светлую колонию, можно вычислить частоту изменений одной клетки за генерацию, разделив найденную величину на предполагаемое число клеток, соответствующее образованию в колонии секторов данного размера. Если найденное число и отличается от истинных значений частоты мутирования, оно все же может служить критерием при изучении влияния различных веществ на скорость мутагенеза.

Для изучения влияния радиозащитных веществ на скорость мутирования Bacl prodigiosum эти вещества добавлялись в расплавленный агар, на котором впоследствии проходил подсчет секторов у выросших колоний. Часть веществ обладала токсическими свойствами, и поэтому опытным путем определялась наименьшая токсическая концентрация (в данных условиях), и затем употреблялись субтоксические разведения. Наличие антамутагенного действия проверялось у гидросульфита натрия, цистеина, унитиола (отечественный препарат, близкий по составу к импортному 2,3-димеркаптопропанолу-препарату БАЛ) и тиомочевины. Кроме того, был испытан «заведомый» мутаген — перманганат калия.

При подсчете частоты мутирования на генерацию изучение колоний производилось с помощью бинокулярной лупы. Учитывались сектора, занимающие 1/2, 1/4, 1/8 и в некоторых опытах 1/16 площади колонии. Сектора подсчитывались в колониях, лежащих изолированно и имевших не менее 3—5 *мм* в диаметре. За статистическую единицу принимался результат подсчета секторов на 25—30 колониях, растущих на одной чашке.

Данные с количеством опытов, меньшим 30, обрабатывались по формулам для малых выборок. Оценка достоверности различий проводилась с коэффициентом вероятности, равным 0,95.

Результаты

Вычисление скорости мутирования описанным способом дало величины, приведенные в таблице в графе «контроль». Частоты мутагенеза

Действие гидросульфита натрия, тиомочевины и перманганата калия на частоту мутирования штамма Б, Bacl. prodigiosum

Размер сектора	Частота мутирования за генерацию			
	контроль	гидросульфит натрия (0,05%)	тиомочевина (0,025%)	перманганат калия (0,0005%)
1/2	$10{,}0 \pm 1{,}6$ $\sigma = 3{,}2$ $n = 20$	$0{,}8 \pm 0{,}35$ $\sigma = 1{,}1$ $n = 36$	$7{,}0 \pm 1{,}3$ $\sigma = 3{,}4$ $n = 27$	$15{,}2 \pm 1{,}8$ $\sigma = 5$ $n = 30$
1/4	$7{,}6 \pm 0{,}8$ $\sigma = 1{,}6$ $n = 20$	$0{,}7 \pm 0{,}23$ $\sigma = 0{,}7$ $n = 36$	$3{,}9 \pm 0{,}57$ $\sigma = 1{,}4$ $n = 27$	$9{,}5 \pm 1{,}1$ $\sigma = 2{,}9$ $n = 30$
1/8	$6{,}7 \pm 0{,}8$ $\sigma = 1{,}7$ $n = 20$	$0{,}7 \pm 0{,}13$ $\sigma = 0{,}45$ $n = 36$	$4{,}0 \pm 0{,}57$ $\sigma = 1{,}4$ $n = 27$	$8{,}4 \pm 0{,}8$ $\sigma = 2{,}1$ $n = 30$
1/16	$6{,}7 \pm 0{,}06$ $\sigma = 0{,}1$ $n = 20$	—	—	—

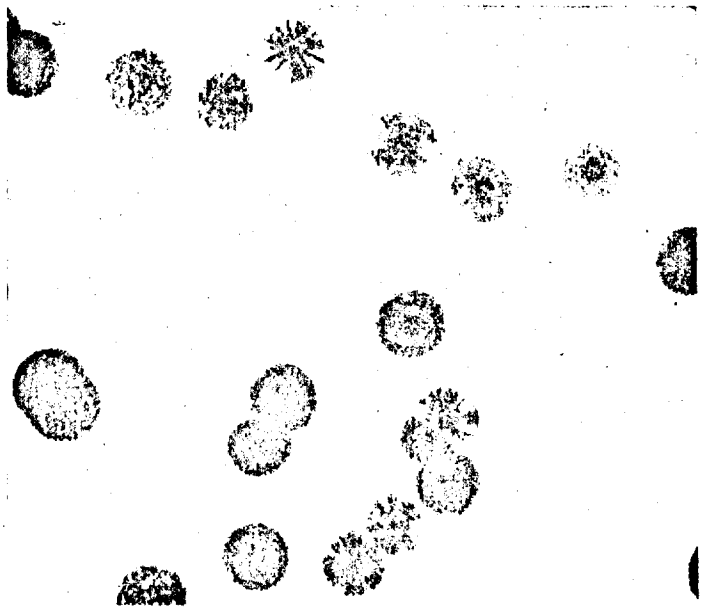

Рис. 2. Светлые и темные колонии нестабильного штамма $Б_1$ Bact. prodigiosum

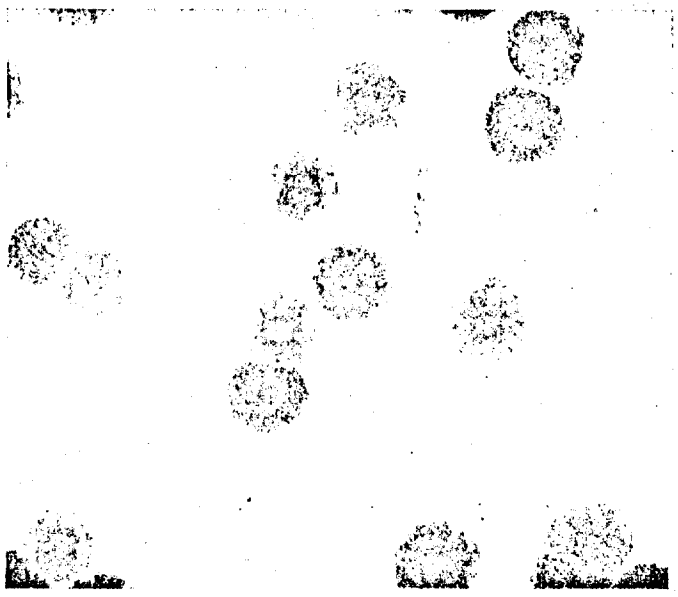

Рис. 3. Светлые и темные колонии нестабильного штамма $Б_1$ Bact. prodigiosum на сахарном агаре с 0,05% гидросульфит. натрия

за генерацию, подсчитанные для секторов каждого класса, довольно близко совпадали (кроме секторов, занимающих половину площади колонии). Получение несколько больших цифр в последнем случае объясняется, возможно, тем, что в числе колоний-«половинок» дополнительно были и колонии, выросшие не из одной, а из двух «слившихся» в момент посева клеток — «светлой» и «темной».

Результаты воздействия радиозащитных веществ на скорость мутагенеза приведены в той же таблице. Гидросульфит натрия и гнмочевина замедляли естественный мутагенез. В особенности заметное действие оказывал гидросульфит натрия, замедляя частоту мутирования более чем в 10 раз. Светлые колонии, растущие на агаре с гидросульфитом натрия, имели лишь очень узкие темные сектора и казались почти сплошными (рис. 3).

Между скоростью мутагенеза в контроле и в присутствии уинтнола (0,5%) и цистеина (0,01%) статистически достоверных различий не было. Поэтому эти данные в таблицу не помещены. Перманганат калия усиливал мутагенез.

Результаты, полученные по действию испытанных веществ, формально могут быть объяснены не только замедлением или ускорением мутагенеза, но и частичной селекцией «светлых» или «темных» клеток. Однако если бы в этих опытах происходило частичное торможение роста или частичное отмирание определенного рода клеток («темных»), то темные колонии на агаре с гидросульфитом натрия были бы значительно меньше, чем светлые. Между тем, величина всех колоний была одинаковой и уменьшалась лишь площадь секторов. Следовательно, мы имели дело с истинным замедлением мутагенеза.

Обсуждение результатов

Данные наших опытов не говорят об обязательной связи радиозащитного действия вещества с антимутагенным эффектом. Тем не менее, само по себе обнаружение веществ, обладающих антимутагенным действием, не лишено интереса. По торможению естественного мутагенеза химическими веществами в настоящее время имеются очень немногочисленные данные: работа Новика и Сциллларда (Novick, Szillard, 1952) о замедлении мутагенеза кишечной палочки с помощью аденовина. Наличие антимутагенного действия (по отношению к естественному мутагенезу) у некоторых радиозащитных веществ, кроме теоретического, возможно, будет иметь и практическое значение. До сих пор усилия генетиков были направлены на способы увеличения естественной изменчивости с целью получения новых полезных признаков. Однако в некоторых случаях было бы целесообразным замедлить эту изменчивость (например, диссоциацию микроорганизмов в производственных условиях).

Выводы

1. Была изучена частота мутирования нестабильного штамма Bact. prodigiosum без каких-либо искусственных воздействий и под действием химических веществ.

2. Некоторые радиозащитные вещества (гидросульфит натрия, тиомочевина) уменьшали скорость естественного мутагенеза. Унитиол и цистеин не оказывали такого действия. Один из мутагенов (перманганат калия) увеличивал скорость мутирования.

1-й Московский медицинский институт
им. И. М. Сеченова

Получено 18.XII.1959 г.

ЛИТЕРАТУРА

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THE EFFECT OF SOME RADIATION PROTECTING COMPOUNDS ON THE MUTATION RATE OF THE UNSTABLE STRAIN OF BACTERIUM PRODUGIOSUM

A. A. Prosorov

1. A study was carried out of the mutation rate of the unstable strain of Bact. prodigiosum with no artificial treatment and under the influence of chemical compoands.

2. Certain radiation protecting compounds such as sodium hydrosulphite or thiourea reduce the rate of natural mutagenesis. Unithiol and cysteine do not exert such an effect. One of the mutagens (potassium permanganate) increased the mutation rate.

Enzymologia, 40(6): 360-368, 1971

EFFECT OF DITHIONITE ON ENZYME ACTIVITIES IN VIVO

BY

RAIMO RAUNIO and ESA-MATTI LILIUS

(Dept. of Biochem., Univ. of Turku, Turku 2, Finland)

(with 10 figs.)

(I—XII—1970)*

1. Introduction

It has been shown that certain electron donors and acceptors have an activating or inactivating effect on enzyme activities in vitro (JANICKI[1], ITOH, KAYASHIMA and FUJIMI[2], NAKAGAWA and PERLMANN[3], TONO[4], SIZER and TYTELL[5]). In this laboratory we have previously studied the effect of electron donors and acceptors on alcohol dehydrogenase activity in vivo during the growth cycle of *Escherichia coli* (RAUNIO and LILIUS[6]). We have noticed that alcohol dehydrogenase was activated by electron donors and inactivated by electron acceptors during the growth cycle. A correlation was also found between the k value, which expresses how much energy will be needed to remove an electron from a molecule, and the activity of this enzyme.

The purpose of the present study was to find out whether electron donors activate other enzymes in vivo during the growth of *E.coli*, too. For this purpose we have tested isocitrate dehydrogenase (EC 1.1.1.42), leucine aminotransferase (EC 2.6.1.1.), NADH oxidase (EC 1.6.99.3) and alanine aminopeptidase.

2. Materials and Methods

The organism, its culture, and the preparation of the enzyme extract have been described in our previous paper (RAUNIO and LILIUS[6]). The methods for assays of isocitrate dehydrogenase (OCHOA[7]), leucine aminotransferase (RAUNIO[8]), and alanine aminopeptidase (GOLDBARG and RUTENBERG[9]), and determination of protein (HEINONEN[10]) are to be found in the references cited. In the NADH oxidase assay the sample cuvettes contained 0.5 μmol of NADH in 1.0 ml of 0.1 M potassium phosphate buffer of pH = 7.0, and 0.05 ml of enzyme preparation. The blank cuvettes did not contain NADH.

* Accepted for Publication: 21—1—1971.

3. Results

3.1. Isocitrate dehydrogenase

The specific activity of isocitrate dehydrogenase increased sharply in the acceleration phase of growth of *E.coli*. In normal growth the specific activity was highest at the beginning of the logarithmic phase and decreased at the end of the logarithmic phase. After addition of sodium dithionite to a concentration of 5 mM $Na_2S_2O_4$ to the growth medium when the specific activity was highest, the activity was maintained at a high level. Even 18 h after addition of dithionite, there was no significant decrease in the specific activity (Fig. 1).

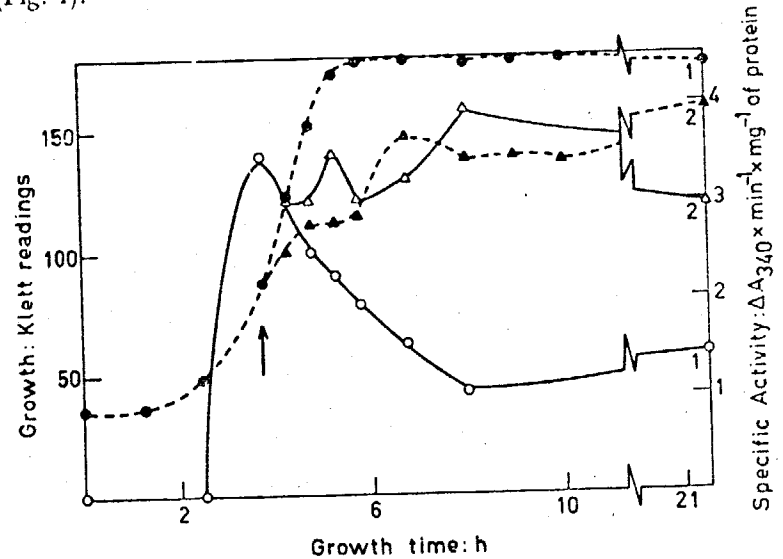
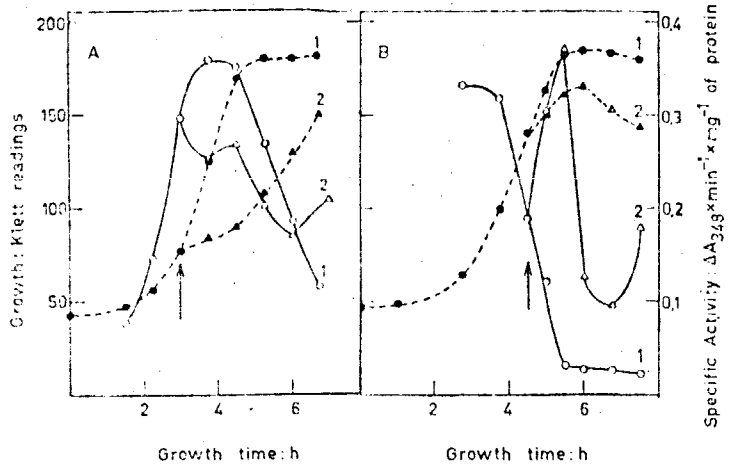


Fig. 1. Effect of $Na_2S_2O_4$ on isocitrate dehydrogenase in vivo during the growth cycle of *E.coli*.
Addition of dithionite to a concentration of 5 mM to the growth medium was made at the time indicated by the arrow.
Solid symbols = growth curves, open symbols = specific activity of isocitrate dehydrogenase.
1 = no addition, 2 = addition of dithionite.



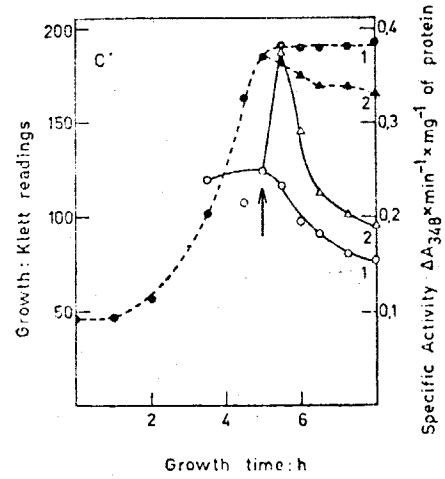
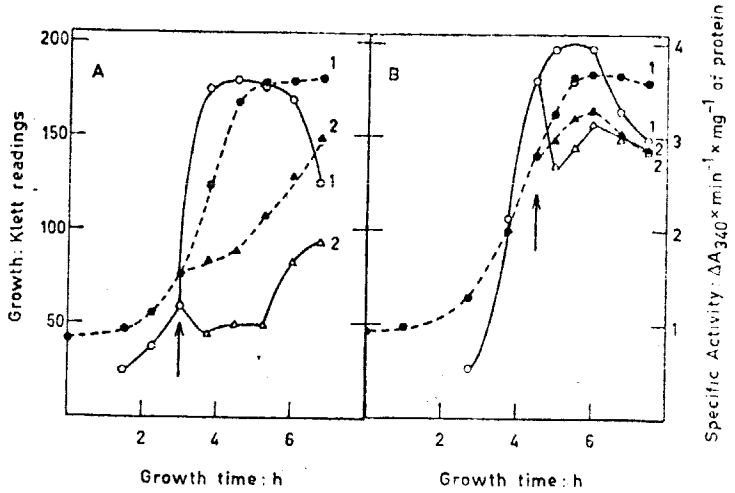


Fig. 2A, B, C. Effect of $Na_2S_2O_4$ on leucine aminotransferase in vivo during the growth cycle of *E.coli*.
Addition of dithionite to a concentration of 5 mM to the growth medium was made at 3 different times (A, B and C), indicated by the arrows.
Solid symbols = growth curves, open symbols = specific activity of leucine aminotransferase.
1 = no addition, 2 = addition of dithionite.



Figs.3A, B, C. Effect of $Na_2S_2O_4$ on NADH oxidase in vivo during the growth cycle of *E.coli*.
Addition of dithionite to a concentration of 5 mM to the growth medium was made at 3 different times (A, B and C), indicated by the arrows.
Solid symbols = growth curves, open symbols = specific activity of NADH oxidase.
1 = no addition, 2 = addition of dithionite.

3.2. Leucine aminotransferase

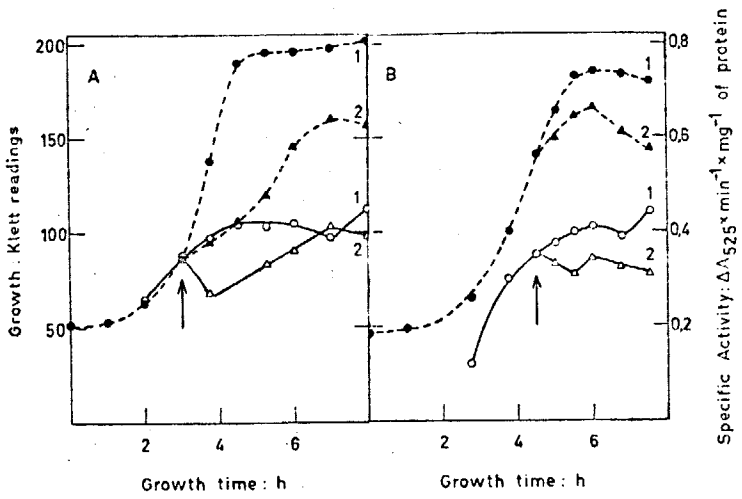
The specific activity of leucine aminotransferase was highest at the end of the logarithmic phase and decreased in the stationary phase in the normal culture. After addition of dithionite to a concentration of 5 mM to the growth medium at the beginning of the logarithmic phase, the specific activity did not rise as high as in normal growth (Fig. 2A). When addition was made later, there was a sharp increase in the specific activity, after which it decreased, although not so much as in normal growth (Figs. 2B and C2).

3.3. NADH oxidase

Fig. 3 shows the results of experiments in which sodium dithionite was added at 3 different points during the growth cycle and the effect of these additions on NADH oxidase activity. After addition of dithionite to a concentration of 5 mM to the growth medium at 3 different growth phases, the specific activity was found to be lower than in normal growth. The later the addition was made, the smaller was the difference between the normal activity curve and the activity curve affected by dithionite (Figs. 3A, B and C on p. 364).

3.4. Alanine aminopeptidase

The specific activity of this enzyme did not decrease at the end of growth in the normal culture. After addition of dithionite, the specific activity decreased for a short time and then increased again. There was no significant difference between the normal activity curve and the activity curve which followed from the addition of dithionite at the beginning of the stationary phase (Figs. 4A, B and C on p. 365).



Figs. 4A, B, C. Effect of $Na_2S_2O_4$ on alanine aminopeptidase in vivo during the growth cycle of *E.coli*.
Addition of dithionite to a concentration of 5 mM to the growth medium was made at 3 different times (A, B and C), indicated by the arrows.
Solid symbols - - growth curves, open symbols — specific activity of alanine aminopeptidase.
1 = no addition, 2 = addition of dithionite.

3.5. Addition of KNO_3 to the growth medium

We also added an electron acceptor, KNO_3, to a concentration of 50 mM to the growth medium of *E.coli* at the end of the logarithmic phase of growth. It had no effect on enzyme activities.

4. Discussion

In previous papers on this subject it has been assumed that the sensitivity of enzymes to the redox state in vitro is due to the oxidation and reduction of the sulphydryl groups in the enzyme molecule (JANICKI[1], ITOH, KAYASHIMA and FUJIMI[2], NAKAGAWA and PERLMANN[3], TONO[4], SIZER and TYTELL[5]). In a recent study (RAUNIO and LILIUS[6]), we have shown that electron donors, such as certain amino acids, purines and pyrimidines, activate alcohol dehydrogenase during the growth of *E.coli*. We assumed that the environment can remove electrons from the enzyme molecule, causing inactivation. Groups on the enzyme molecule that might be labile in this respect are, in addition to sulphydryl groups, tryptophan, phenylalanine, tyrosine and histidine. Enzyme inactivation can be prevented by adding these electron-donating compounds to the growth medium.

The explanation offered to account for the results presented here is the effect of the redox state in vivo is specific to each enzyme. The activation of isocitrate dehydrogenase, like that of alcohol dehydrogenase, is irreversible. In these enzymes the specific activity does not decrease at the end of growth after addition of dithionite, as in normal growth without addition of an electron donor (Fig. 1). Leucine aminotransferase is also activated by dithionite, but after a short time inactivation begins almost normally (Fig. 2C). The decrease of the specific activity of NADH oxidase is increased by dithionite (Fig. 3C). Alanine aminopeptidase is not significantly affected by this compound (Fig. 4C).

If dithionite is added before the specific activity peak of these enzymes is reached, the specific activity of each enzyme remains lower than normal (Figs. 2A, 3A and 4A). We think that dithionite inhibits the synthesis of these enzyme proteins. This is supported by the fact that growth is slower in culture containing dithionite. Dithionite could inactivate enzymes involved in protein synthesis in the same way as it inactivates some of the enzymes investigated in this paper (Figs. 3 and 4). In addition, in our earlier paper we showed that purines and pyrimidines protected alcohol dehydrogenase against inactivation and we assumed that these compounds donate electrons to the environment (RAUNIO and LILIUS[6]). They can do this within the DNA molecule, too, thus inhibiting protein synthesis somehow.

Thus our conclusion is that when dithionite is added to the growth

medium before the synthesis of a particular enzyme is proceeding at the maximal rate, the activity of this enzyme remains lower because of inhibition of its synthesis by dithionite. On the other hand, when the addition is made at the peak of activity of a particular enzyme, the enzyme may be either inactivated or activated by dithionite, depending on the individual properties of the enzyme in question. This inactivation or activation might be due to the presence of labile groups in the enzyme molecule which are affected by electron donors and acceptors.

Summary

The specific activity of isocitrate dehydrogenase, leucine aminotransferase, NADH oxidase and alanine aminopeptidase was investigated after the addition of an electron donor, sodium dithionite, to the concentration of 5 mM to the growth medium of *Escherichia coli*. The specific activity of each enzyme was lower than normal when the addition was made before the activity reached its maximum. When the addition was made during this maximum, the loss of the specific activity of isocitrate dehydrogenase and leucine transaminase was decreased. NADH oxidase and alanine aminopeptidase were, however, inactivated by dithionite when added at that stage. An electron acceptor, potassium nitrate, added at the end of the growth to the medium, had no effect on the activity of these enzymes.

Zusammenfassung

Die spezifische Aktivität von Isocitrat-Dehydrogenase, Leucin-Aminotransferase, NADH-Oxydase und Alanin-Aminopeptidase wurde nach dem Zusatz eines Elektronendonators, Natriumdithionit, in einer Konzentration von 5 mM zu dem Züchtungsmedium von *Escherichia coli* untersucht. Wenn der Zusatz vor dem Aktivitätsmaximum gemacht wurde, war die spezifische Aktivität jedes Enzyms niedriger als ohne Zusatz. Wenn der Zusatz während dieses Maximums gemacht wurde, fiel die spezifische Aktivität von Isocitrat-Dehydrogenase und Leucin-Aminotransferase langsamer als ohne Zusatz. Dagegen wurde NADH-Oxydase und Alanin-Aminopeptidase von Dithionit inaktiviert, auch wenn es in diesem Stadium zugesetzt wurde.

Ein Elektronenakzeptant, Kaliumnitrat, hatte kein Effekt auf der Aktivität dieser Enzyme, wenn der Zusatz gegen Ende der Züchtung gemacht wurde.

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INDEPENDENCE MALL WEST, PHILADELPHIA, PA 19105 TELEPHONE (215) 592-3000

June 21, 1973

Mr. Frank J. Letkiewicz
Tracor Jitco, Inc.
1300 E. Gude Drive
Rockville, Maryland 20851

Dear Mr. Letkiewicz:

Thank you for your letter of June 14, 1973 requesting information on sodium hydrosulfite (Lykopon SF) and zinc hydrosulfite (Protolin Z).

These products have GRAS approval for can coatings, adhesives, paper and paperboard applications. As for food uses, these products are not food grade and do not have FDA clearance for this application. I have enclosed our technical bulletin discussing these products for your review.

I hope this information proves satisfactory and thank you for this opportunity to have been of service.

Sincerely,

L. C. Newman

Leonard C. Newman, Marketing Specialist
Organic and Inorganic Chemicals
Industrial Chemicals Department

LCN/lf
Enclosure

ROHM AND HAAS COMPANY
INDEPENDENCE MALL WEST
PHILADELPHIA, PENNSYLVANIA 19105

HYDROSULFITES AND SULFOXYLATES

A. INTRODUCTION

Metal hydrosulfites and sulfoxylate formaldehydes are chemicals characterized by their high reducing capacity. Four such compounds manufactured by Rohm and Haas Company are

LYKOPON (sodium hydrosulfite)

PROTOLIN Z (zinc hydrosulfite)

~~FORMOPON (sodium sulfoxylate formaldehyde)~~ DISCONTINUED

~~FORMOPON EXTRA (basic zinc sulfoxylate formaldehyde)~~ DISCONTINUED

In general, the hydrosulfites and sulfoxylate formaldehydes are used primarily as reducing agents and bleaching agents. Because of variations in stability at different temperature ranges and pH values, however, some of these compounds are more adaptable to certain applications and have become established reagents in special fields. For example, LYKOPON and FORMOPON are widely used as reducing agents in redox emulsion polymerizations of acrylic monomers and in rubber formulations. They are also employed extensively in textile manufacturing for the reduction of vat dyes to soluble forms, for stripping dyestuffs from cloth, and for bleaching textiles. All four reducing agents are utilized in bleaching wood pulp, soap, sugar, molasses, glue, and oils and fats.

B. PHYSICAL PROPERTIES

The hydrosulfites and sulfoxylate formaldehydes manufactured by Rohm and Haas Company differ quite widely in physical and chemical properties and in the degree of efficiency at which they operate under specific reaction conditions. This diversity permits a choice of the reducing agent that is most suitable for any given application.

A summary of some of the physical and chemical properties of LYKOPON, PROTOLIN Z, FORMOPON, and FORMOPON EXTRA is given in Table I.

CI-30 G/ch
(Supersedes SP-21 8/65)

TABLE I

TYPICAL PROPERTIES OF THE HYDROSULFITES AND SULFOXYLATES

	LYKOPON	PROTOLIN Z	FORMOPON	FORMOPON EXTRA
	Sodium Hydrosulfite	Zinc Hydrosulfite	Sodium Sulfoxylate Formaldehyde	Basic Zinc Sulfoxylate Formaldehyde
Formula	$Na_2S_2O_4$	ZnS_2O_4	$NaHSO_2 . CH_2O . 2H_2O$	$Zn(OH)HSO_2 . CH_2O$
Molecular Weight	174.1	193.5	154.1	177.5
Equivalent Weight[a]	87.0	96.7	77.0	88.7
Form	Free flowing crystalline powder	Free flowing non-dusting powder	Pea size, grain size, and powder; crystalline	Fine dry powder
Particle Size	100% passes through U.S. Bureau of Standards #24 mesh screen.	1.0% max. retained on U.S. Bureau of Standards #20 screen.	Pea: 8 mesh to 1/2" Grain: 65% min. passes U.S. Bureau of Standards #8 but retained on U.S. Bureau of Standards #30 screen. Powder: 95% min. passes U.S. Bureau of Standards #10 screen.	1.0% retained on U.S. Bureau of Standards #30 screen.
Purity, %	94 min.	84-87 min.[b]	Pea: 99.0-101.0[c] Grain & Powders: 97.0-101.0[c]	88-90 min.
Color	White	White	White	Light gray
Odor	Free of H_2S and SO_2	Slight SO_2	Odorless to faint garlic-like	Faint formaldehyde odor
Suggested pH for Use	7.5-11.0	5.5-7.0	3.2-3.5[d]	3.2-3.5[d]
Solubility in Water, oz. per gal. at 86°F.	31	49	86	0.1
Other Properties	Stable in alkali. Decomposes in acid.	Some tubidity in aqueous solution.	Decomposes slowly in warm, moist air.	Soluble in acid solutions under pH 4.0

a - Based on ammoniacal copper method. Refer to Am. Dyestuff Reptr. 46, 443-447 (1957).
b - 84.0% ± 1.0% by Iodine Method; 87.0% ± 1.0% by Rubine R Method. Refer to Am. Dyestuff Reptr. 46, 443-447 (1957).
c - Traces of water of crystallization are driven off in manufacture; thus, apparent purity may exceed 100%.
d - It is advisable to agitate the solution during acidification to prevent local concentration of acid and subsequent undue decomposition.

1. EFFECT OF pH

The effective reducing capacity of the hydrosulfites and sulfoxylate formaldehydes is directly related to the pH at which these compounds are used. Suggested pH ranges for the most efficient and economical operation are given in Table I for each of the reducing agents. In very alkaline systems, all of these products are quite stable and afford little, if any, reducing activity. As the pH is lowered the activity increases. However, if the pH is lowered appreciably below the suggested range, the material will decompose so rapidly that much of the reducing activity is lost. In the case of LYKOPON (sodium hydrosulfite), the most effective and economic reducing action is obtained at a pH of 7.5-11. PROTOLIN Z (zinc hydrosulfite) is used most effectively at a pH of 5.5-7.0. The sulfoxylate derivatives, FORMOPON and FORMOPON EXTRA, are much more stable and the pH must be lowered to about 3.2-3.5 for the best results. It is important for the solution to be agitated while the pH is being lowered because local concentrations of acid cause rapid decomposition, which is undesirable.

2. EFFECT OF TEMPERATURE

The reactivity of the hydrosulfites and sulfoxylate formaldehydes is affected by temperature - the higher the temperature, the greater the reducing power. At lower temperatures, the reduction potential is lower but is exerted over a longer period of time. At any given temperature, the total reducing power and available reduction time is dependent upon the amount of hydrosulfite or sulfoxylate present. LYKOPON, the most reactive of the Rohm and Haas reducing agents, is most efficient at approximately 120°F. PROTOLIN Z is effective at 100-170°F., with the higher temperatures preferred for most economical operation. FORMOPON and FORMOPON EXTRA can be used at temperatures of 180-212°F. FORMOPON EXTRA is the most stable of the four reducing agents and is often utilized at the upper end of the temperature range, 212°F.

C. TOXICITY

HYDROSULFITES

Acute Oral Toxicity - May be tolerated in fairly large doses since they are rapidly oxidized to sulfates. The alimentary tract, however, may be irritated during ingestion.

Skin Irritation - May be irritating to the skin and mucous membranes.

Inhalation Toxicity - Inhalation of the dust should be avoided.

ANTIDOTE STATEMENT: If hydrosulfites are taken internally, vomiting should be induced by inserting finger into throat, copious quantities of water consumed and medical attention obtained for gastric lavage. In case of contact with skin, exposed areas should be washed with soap and water. In case of contact, the eyes should be flushed with copious amounts of water for 15 minutes. If irritation persists, an ophthalmologist should be consulted. Breathing of dust should be avoided. Clothing should be washed before re-use.

SODIUM SULFOXYLATE FORMALDEHYDE

Acute Oral Toxicity - Rats: LD = > 1.00 g. per kg. body wt. (1)
Rabbits: LD = > 1.00 g. per kg. body wt. (1)
(No symptoms were observed at this dosage except slight diarrhea in rats.)

Skin Irritation - Not expected to be a skin irritant.

Inhalation Toxicity - Inhalation of the dust should be avoided.

ANTIDOTE STATEMENT: If sodium sulfoxylate formaldehyde is taken internally, vomiting should be induced by inserting finger into throat, copious quantities of water consumed and medical attention obtained for gastric lavage. In case of contact with skin, exposed areas should be washed with soap and water. In case of contact, the eyes should be flushed with water. Breathing of dust should be avoided.

D. REDUCING POWER

The reducing power of LYKOPON, PROTOLIN Z, FORMOPON, and FORMOPON EXTRA, in terms of pounds of material for equivalent activity, is given in Table II.

TABLE II

COMPARISON OF REDUCING POWER OF THE HYDROSULFITES AND SULFOXYLATES

Product	Molecular Weight	Equivalent* Weight	Purity %	Pounds of Material For Equivalent Activity**
LYKOPON (sodium hydrosulfite)	174.1	87.0	94 min.	1.4
PROTOLIN Z (zinc hydrosulfite)	193.5	96.7	84-87 min.	1.8
FORMOPON (sodium sulfoxylate formaldehyde)	154.1	77.0	99-101 min.	1.2
FORMOPON EXTRA (basic zinc sulfoxylate formaldehyde)	177.5	88.7	88-90 min.	1.5

* Based on ammoniacal copper method.

** The weights given above are calculated on the basis of optimum pH and temperature for each of the reducing agents. In substituting a chemically equivalent amount of one reducing agent for another, the effective reducing power may appear to vary depending upon the reaction conditions used. Thus, the reducing activity of 1.4 pounds of LYKOPON would appear to be less than that of 1.8 pounds of PROTOLIN Z if LYKOPON were used at the higher temperatures (ca. 170°F.) where PROTOLIN Z is often employed.

E. APPLICATIONS

The hydrosulfites and sulfoxylates have been studied and used in a wide variety of applications on a world-wide basis since before 1900. Unlike many other chemicals, they have not been replaced by newer reducing agents. Rather, the purer grades such as those offered by Rohm and Haas offer greater advantages over those formerly available. Some of the applications in which the hydrosulfites and sulfoxylates are used and a few of the many references pertaining to these products are given below.

1. EMULSION POLYMERIZATION

The hydrosulfites and sulfoxylates have attained importance as reducing agents in the preparation of emulsion polymers.

Emulsion polymerization is most commonly a free radical polymerization initiated by the decomposition of a catalyst into free radicals. To use a catalyst such as a peroxide, persulfate, or similar material with a reasonable storage life at room temperatures to initiate emulsion polymerization, either (1) the polymerization must be carried out at elevated temperatures, or (2) decomposition of the catalyst must be induced by the use of a reducing agent. The second method, a redox catalyst system, has the advantage of providing a rapid polymerization rate without external heating. This results in savings in equipment costs since reflux condensers and external heaters may not be needed. There may also be some savings in catalyst costs. LYKOPON and FORMOPON are reducing agents excellently suited for use in redox catalyst systems in emulsion polymerization*.

A typical recipe for an emulsion polymerization would require about 0.5%, based on the weight of the monomer, of a catalyst e.g. ammonium persulfate, plus approximately an equivalence of LYKOPON or FORMOPON. The actual range of ratios of reducing agent to catalyst to achieve optimum polymerization rates may be limited to a narrow range which would vary with the monomers used. This range of optimum reducing agent to catalyst ratio is probably dependent upon the initial temperature of the system. Too high a ratio would dissipate the catalyst before a high degree of monomer conversion has been reached; at lower ratios the amount of catalyst decomposed by the reducing agent may not be sufficient to initiate polymerization.

It is usually good practice to initiate a redox polymerization at as low a temperature as is practical since an exotherm of 60-65°C. is not uncommon; higher initial temperatures would allow the exotherm to bring the temperature to the refluxing point. This would dissipate the catalyst before the reaction has reached a high degree of conversion. It is important that sufficient water be present in the emulsion system to dissipate the heat produced, thereby preventing the system from reaching reflux temperature.

Heavy metals in low concentrations (1-15 ppm) act as promoters for some redox catalyst systems and may greatly increase the rate of polymerization. Cupric, ferric, chromic, and silver salts are effective. On the other hand, excessive amounts of heavy metals will often inhibit polymerization; therefore, polymerization should be conducted in stainless steel or glass-lined kettles. Distilled or deionized water should be used to assure consistent results.

The use of FORMOPON and LYKOPON in redox emulsion polymerizations is of particular interest in at least two fields - acrylic emulsion polymers and cold rubber (butadiene-styrene) emulsion polymers.

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* A detailed discussion is presented in CM-104 (Emulsion Polymerization of Acrylic Monomers), published by Rohm and Haas Company, Industrial Chemicals Department.

ACRYLIC EMULSION POLYMERS

FORMOPON and LYKOPON are equally effective in both single-stage and two-stage acrylic redox emulsion polymerizations. Because of the rapid rate of decomposition of the catalyst in such systems, however, the catalyst is often depleted before the polymerization is complete. In order to take the reaction as near to completion as possible, a small amount (about 0.01% based on the weight of monomer) of a second catalyst having a long half-life at low temperatures is also added. An example of such a catalyst is t-butyl hydroperoxide.

Typical recipes and laboratory procedures for a single-stage and a two-stage redox acrylic emulsion polymerization are given below. In the single-stage recipe, TRITON X-200* is used as the emulsifier. In the two-stage system, the sodium salt of TRITON QS-44** is the emulsifying agent in both stages.

1. SINGLE-STAGE REDOX RECIPE

A. PROCEDURE

MATERIALS

Deionized Water	376	g.
TRITON X-200	24	g.
Ethyl Acrylate (15 ppm MEHQ)	120	g.
Methyl Methacrylate (10 ppm MEHQ)	80	g.
Methacrylic Acid (100 ppm MEHQ)	2	g.
$FeSO_4$ Solution (0.3 g. $FeSO_4 .7H_2O$/200 g. H_2O, freshly prepared), representing 2 ppm of iron in the emulsion	4	g.
Ammonium Persulfate	1	g.
FORMOPON	0.7	g.
t-Butyl Hydroperoxide (70%)	0.25	g.

A 1-liter 3-neck flask is rinsed with deionized water and equipped with a 3-inch Teflon paddle stirrer capable of rotating at approximately 250 rpm, a thermometer, a water-cooled reflux condenser, and a nitrogen inlet tube extending close to the bottom wall of the flask. Deionized water (376 g.) and TRITON X-200 (28% solids) (24 g.) are added and the stirrer is started. After the surfactant has been completely dissolved, ethyl acrylate (120 g.), methyl methacrylate (80 g.), and methacrylic acid (2 g.) are added slowly in sequence to form an emulsion. The freshly prepared ferrous sulfate solution (4 g.) is added and stirring is continued for 15 minutes during which nitrogen is fed through the dip tube at approximately 50 cc. per minute. The temperature of the mixture is then adjusted to 20°C. and ammonium persulfate (0.7 g.), FORMOPON (0.7 g.), and t-butyl hydroperoxide (70%) (0.25 g.) are added. After a 5-minute induction period the temperature rises to approximately 80°C. within 2-3 minutes. Stirring is continued for 15 minutes and the emulsion is then cooled to room temperature and strained through cheesecloth.

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* Refer to Rohm and Haas Company, Industrial Chemicals Department Bulletin CS-25 (TRITON X-200 and TRITON X-202).

** For additional information on the use of TRITON QS-44 as a principal surfactant in emulsion polymerizations, see Bulletin CS-34 (Phosphate Ester Surfactants) available from Rohm and Haas Company, Industrial Chemicals Department.

B. PROPERTIES OF SINGLE-STAGE EMULSION

Some typical properties of the single-stage emulsion prepared above are listed below.

Solids (calculated), %	34.3
Solids (found), %	34.3
pH of Emulsion Polymer at 26°C.	2.6
Viscosity, cps, (Brookfield)	6.0
Minimum Filmforming Temperature (MFT), °C.	5.0
Tukon Film Hardness (KHN units)	1.99

2. TWO-STAGE REDOX RECIPE

A. PROCEDURE

MATERIALS	FIRST STAGE	SECOND STAGE
Deionized Water	375 g.	90 g.
TRITON QS-44	5.1 g.	5.1 g.
Ethyl Acrylate (15 ppm MEHQ)	100 g.	100 g.
Methyl Methacrylate (10 ppm MEHQ)	100 g.	100 g.
Methacrylic Acid (100 ppm MEHQ)	4 g.	4 g.
$FeSO_4$ Solution (0.3 g. $FeSO_4 \cdot 7H_2O$/200 g. H_2O, freshly prepared)	4 g.	4 g.
Ammonium Persulfate in 5 cc. H_2O	1 g.	1 g.
FORMOPON in 5 cc. H_2O	0.7 g.	0.7 g.
t-Butyl Hydroperoxide	5 drops	5 drops

1. FIRST STAGE

Into a thoroughly clean beaker (washed with deionized water) is measured TRITON QS-44 (80% solids) (5.1 g.) and deionized water (375 g.) and the mixture is stirred with a magnetic stirrer until the surfactant is completely dissolved. The solution is adjusted to pH 9.0 with 50% sodium hydroxide solution using a calibrated pH meter. The solution is then charged to a 1-liter 3-neck flask (rinsed with deionized water) equipped with a 3-inch Teflon paddle stirrer rotating at approximately 250 rpm, thermometer, nitrogen inlet tube extending beneath the surface of the liquid, and reflux condenser. A solution of the three monomers (ethyl acrylate, 100 g., methyl methacrylate, 100 g., and methacrylic acid, 4 g.) is then added to the mixture in the reaction flask. After stirring to form an emulsion, the freshly prepared ferrous sulfate solution (4 g.) is added and the mixture is stirred for 15 minutes with nitrogen being fed in through the dip tube at approximately 50 cc. per minute. The temperature is then adjusted to 20°C. and ammonium persulfate (1.0 g. dissolved in 5 cc. deionized water), FORMOPON (0.7 g. dissolved in 5 cc. deionized water), and t-butyl hydroperoxide (5 drops) are added. The cooling bath is removed and the temperature is recorded every minute until the maximum temperature is reached. The time required to reach the maximum temperature (ca. 77°C.) is usually 12-15 minutes. After the temperature begins to drop, stirring is continued for 15 minutes and the emulsion is then cooled to 20-25°C.

2. SECOND STAGE

The second emulsion is made as follows: TRITON QS-44 (80% solids) (5.1 g.) and deionized water (90 g.) are mixed thoroughly until the surfactant is completely dissolved. The pH of the mixture is then adjusted to 9.0 with 50% sodium hydroxide solution as in the first stage. The mixture is transferred to a 500 ml. flask, previously rinsed with deionized water, and ethyl acrylate (100 g.), methyl methacrylate (100 g.), methacrylic acid (4 g.), and ferrous sulfate solution (4 g.) are added. The mixture is then shaken vigorously until a homogeneous monomer emulsion is formed.

The monomer emulsion is added to the first stage emulsion polymer with stirring. Stirring is continued for 15 minutes while the mixture is purged with nitrogen and the temperature is adjusted to 20°C. The ammonium persulfate (1 g. in 5 cc. water), FORMOPON (0.7 g. in 5 cc. water), and t-butyl hydroperoxide (5 drops) are added in succession, the cooling bath is removed, and the temperature is recorded every minute until the maximum temperature (ca. 65°C.) is reached (ca. 7-10 minutes). Stirring is continued for 15 minutes to allow the reaction to go to completion, and the emulsion is then cooled to room temperature and discharged through cheesecloth.

B. PROPERTIES OF TWO-STAGE EMULSION

Typical properties of the emulsions obtained in each stage of the two-stage recipe above are shown below.

	FIRST STAGE EMULSION	TWO-STAGE EMULSION
Solids (calculated), %	35.00	45.91
Solids (found), %	34.31	45.36
% Surfactant Solids Based on Monomer	2.0	2.0
pH of Emulsion Polymer at 25°C.	5.6	5.8
Particle Size as % Light Scatter	22.8	23.6
Viscosity, cps, (Brookfield, 60 rpm)	7.9[a]	35.5[a]
Minimum Filmforming Temperature (MFT), °C.	22[a]	-
Gums, %	0.17	0.25

a - Emulsion adjusted to pH 9.5 with concentrated ammonium hydroxide before measurement was taken.

The preparation of poly(methyl methacrylate) emulsions has been reported in which LYKOPON (2) was used as initiator. Methods employing LYKOPON (3) and FORMOPON (4) for making polyacrylonitrile emulsions have also been published.

COLD RUBBER EMULSION POLYMERS

FORMOPON is important as an activator in emulsion polymerization recipes for the preparation of cold rubber, for example, the 70:30 butadiene:styrene copolymer made at 40°F. (5, 6). Compared with other redox systems, sulfoxylate accelerated systems have the following advantages:

1. Improved polymerization reproducibility.
2. The possibility of appreciable cost savings because of the lower concentrations of organic hydroperoxides required.
3. Improved color of latexes and improved color and aging resistance in polymer vulcanizates because of the lower concentrations of iron compounds needed.
4. Production of high-solids and medium-solids latexes of much lower viscosity.

LYKOPON is also a highly useful reagent in the preparation of cold or GR-S rubber. It can be utilized as a polymerization accelerator (7), as a polymerization stopping agent to terminate chain growth (8), or as an oxygen scavenger in recycled butadiene or in emulsion recipes containing ferrous iron (9).

Additional information on the role of FORMOPON and LYKOPON in cold rubber emulsion polymers is available on request.

OTHER POLYMER SYSTEMS

Numerous examples of other copolymers and homopolymers prepared in the presence of sulfoxylate or hydrosulfite activators are found in the literature. Some of these, listed according to the activators and monomers used, are:

LYKOPON (Sodium Hydrosulfite)

methyl methacrylate-butadiene (2)
methyl methacrylate-vinyl acetate (2)
vinyl acetate (10)
vinyl chloride (2, 10, 11)
vinyl chloride-vinyl acetate (2)

FORMOPON (Sodium Sulfoxylate Formaldehyde)

70:30 butadiene-methyl acrylate (5)
50:50 butadiene-isoprene and polyisoprene (5)
vinyl chloride (4)

In many cases, FORMOPON is the preferred reducing agent because of its relative stability and efficiency around 100°C., the temperature necessary for copolymers having high orientation and toughness. The use of the sulfoxylates or hydrosulfites also reduces the amount of heavy metal ions required (12).

2. TEXTILES

LYKOPON (13) and FORMOPON (14) are used extensively in the textile industry for the reduction of vat dyestuffs to a soluble form which can be picked up by fabrics and subsequently fixed on the cloth. The sulfoxylate formaldehyde is also employed for discharge printing of dyed textiles, preparation of oil-in-water emulsions of textile printing pastes (15), and flash-age fixation of vat prints (16). In the dyeing and printing of cellulose acetate fibers, both FORMOPON and FORMOPON EXTRA (the sodium and basic zinc sulfoxylate formaldehydes) have been recommended (17).

The hydrosulfites and sulfoxylates are also effective agents for removing dyestuffs from textiles when it is desirable to redye or rework the materials (18). FORMOPON EXTRA, particularly, has been used in conjunction with acetic acid (19), citric acid (20), oxalic acid (21) and succinic acid (22) as a color and stain remover for fabrics.

The removal of residual chlorine from hypochlorite-bleached natural fibers (23) and synthetic fibers such as nylon (24, 25) by means of LYKOPON, FORMOPON, and FORMOPON EXTRA is another application of these materials in the textile field.

Literature giving more detailed information on the use of Rohm and Haas reducing agents in the textile field may be obtained on request*.

3. PAPER

PROTOLIN Z, LYKOPON, and FORMOPON** are used to improve the color of mechanical pulp (groundwood) in the manufacture of white and light-colored paper (26, 27).

Compared with other bleaching agents, such as peroxides, treatment with the hydrosulfites offers the advantages of low cost per point of gain in brightness, less manipulation, and the possibility of bleaching in existing or only slightly modified paper mill equipment (28).

In the bleaching of spruce groundwood, southern pine groundwood, and poplar groundwood, PROTOLIN Z is often preferred on the basis of cost. Used at the level of 0.5-1.0%, PROTOLIN Z improves the color of the pulp by several points on the Photovolt brightness scale. The reducing agent may be added to the pulp in the grinder pit or in the beater but the preferred practice is to add it to the stock chest; the last procedure minimizes aeration which decreases the efficiency of the bleaching agent.

Equally good brightening of spruce groundwood (29, 30) and poplar groundwood (31) has also been reported by the use of LYKOPON. In the case of aspen groundwood, laboratory and mill trials indicate that sodium hydrosulfite is a more economical reducing agent than the corresponding zinc salt (32).

LYKOPON is employed widely in the paper industry to strip the color (vat dyes) from cotton rags for the preparation of rag paper. In this process the dyes are reduced to a soluble form and then removed by rinsing with water. As in other reductions, the operation is sensitive to oxygen and should therefore be carried out in a closed vessel for maximum efficiency.

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* Refer to Technical Bulletins T-9b (LYKOPON), T-7b (FORMOPON), and T-6c (Color Stripping with Reducing Agents).

** Copies of Technical Bulletins TP-100 (Hydrosulfites in Bleaching Pulp for Paper) and TP-78 (PROTOLIN Z) are available from Rohm and Haas Company, Philadelphia, Pa. 19105.

4. SOAP

LYKOPON is an inexpensive, practical, and efficient bleach in soap production.

To obtain the best results, LYKOPON must be added to the kettle when the soap mixture contains a high percentage of caustic. On white soaps, it is added on a strong lye change and prior to the soft washing. On yellow soap, it is added on the wash before the finish. In general, LYKOPON is added after all the water has been added and prior to the separation of the soap from the glycerin and salt. The boil should be continued for several hours after the addition of LYKOPON.

The ratio of one pound of LYKOPON per thousand pounds of stock is about the minimum. The actual amount required depends on the quality of the fat used, with a maximum of two pounds per thousand pounds of stock.

Sodium hydrosulfite, in conjunction with an appropriate ultraviolet absorber such as 2-hydroxy-4-methoxybenzophenone, is reported to enhance the light stability of soaps containing antiseptic agents (33).

5. SUGAR

Hydrosulfites, hydrosulfite derivatives, and particularly sodium hydrosulfite (LYKOPON) are established reagents in the manufacture and refining of sugar (34). Their use results in easier processing because of reduced viscosity of the sugar solutions and in products of lighter color (35, 36). Increased yields of crystalline sugar may also be obtained.

6. MOLASSES

In the preparation of molasses, bleaching effects similar to those obtained with sugar are produced by LYKOPON and FORMOPON. Levels of 1-5 pounds per 1,000 gallons of molasses are used. The reducing agent is first dissolved in water and then added to the syrup. LYKOPON gives good bleaching at 120°F., whereas FORMOPON must be heated to 180°F. In general, FORMOPON gives better retention of the bleached color than LYKOPON and also imparts greater clarity and sparkle. It has been found that the best bleaching is obtained if the operation is carried out on the final molasses rather than the original cane syrup. Since there is a possibility of some redevelopment of color, it is desirable to minimize the exposure to air after bleaching.

7. OILS AND FATS

Sodium hydrosulfite and its derivatives have been used for many years to treat animal and vegetable fatty oils, such as corn oil (37), coconut oil (38), and gum-containing oils (glycerides and fatty acid esters) (39), to remove naturally occurring colored compounds and colored substances formed by oxidation (40). The reducing agents are usually added as a 0.01-2.0% aqueous solution to form an emulsion with the oil being treated. After agitating in a closed vessel at elevated temperatures (ca. 50-100°C.), the emulsion is broken and the decolorized oil is separated and recovered.

8. MISCELLANEOUS

The hydrosulfites and sulfoxylates are utilized in many varied applications in which an economical and effective reducing agent is required. Some of these uses and the respective agents are:

Clay, bleaching of	LYKOPON, PROTOLIN Z	(41, 42)
Clay, removal of iron from	LYKOPON	(43)
Glue, bleaching of	all four reagents	
Hair-curling products, formulation of	LYKOPON	(44)
Hair-grooming preparations, improvement of	LYKOPON	(45)
Hides, depilatory aid in tanning of	LYKOPON	(46)
Iron and steel, separation of copper in analysis of	FORMOPON	(47)
Mercuric chloride poisoning, antidote for	FORMOPON	(1, 48)
Monomers, preparation from trans methyl fumarate and epoxidized soya oil	all four reagents	(49)
Ore-separation, depressant to prevent flotation in	LYKOPON, PROTOLIN Z	
Water-softening resins, removal of iron deposits from	LYKOPON*	
Well-producing equipment, anti-corrosion agent for	FORMOPON	(50)

F. STORAGE AND HANDLING

The hydrosulfites and sulfoxylates decompose under the influence of heat or moisture. They should therefore be kept in sealed water-tight containers and stored in a cool dry place. Under such conditions, these products can be stored with negligible loss in purity over a prolonged period.

FORMOPON is slightly hygroscopic and becomes more unstable as moisture is adsorbed. Pea size FORMOPON is more stable than the grain or powder forms, since the degree of hygroscopicity is inversely proportional to the particle size. In cases where there is any question about storage conditions, pea size FORMOPON should be ordered rather than the finer grades.

Greater care in handling is needed with LYKOPON than with the other hydrosulfites and sulfoxylates. On contact with water LYKOPON decomposes quite rapidly, and gases are formed which can ignite spontaneously. For this reason, LYKOPON is classified as a flammable solid and is shipped under the I.C.C. Yellow Caution Label. The Bureau of Explosives restrictions prohibit the shipment of LYKOPON by Railway Express in packages in excess of 100 lbs. LYKOPON cannot be sent through the mail.

When handling LYKOPON, contact with moisture should be avoided and clean dry utensils should be used at all times. Any material that is spilled should be cleaned up, and the site washed with copious amounts of water. Partly used containers should be resealed because an improperly sealed container of LYKOPON represents a definite fire hazard.

When fighting a LYKOPON fire, the burning material should be deluged with water since too little water may be worse than none at all. Carbon dioxide and dry fire extinguishers are valueless since the product provides its own oxygen for combustion.

* Additional information on LYKOPON treatment of iron-fouled water softeners may be obtained from Rohm and Haas Company, Ion Exchange Department

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Detection of Anions with Silver Chromate-Impregnated Paper

J. G. SABO

Active Materials Laboratory, Electric Storage Battery Co., Belleville, New Jersey

Silver chromate-impregnated paper has previously been proposed for the spot test detection of chloride ion (*1*, *2*). The red color changes to white with the conversion to silver chloride. The use of such paper has now been investigated as a possible preliminary test for some common anions. It has been found that in addition to chloride ion a color reaction is secured with azide, bromide, cyanate, cyanide, hexacyanoferrate(II), hexacyanoferrate(III), iodide, sulfide, sulfite, thiocyanate, and thiosulfate ion. The less common ions pentacyanonitrosylferrate(III), dithionate, dithionite, and hypochlorite also give positive tests.

TABLE I. ANIONS GIVING COLOR REACTIONS WITH Ag_2CrO_4 Paper

Ion tested	Spot color	L.L.[b]
N_3^-	white	16.1
Br^-	white	2.[illegible]
Cl^-	white	2.4
OCN^-	white	41.2[c]
CN^-	white	0.[illegible]
$S_2O_6^{2-}$	white	7.2
$S_2O_4^{2-}$	black[a]	1.0
$Fe(CN)_6^{3-}$	yellow	6.[illegible]
$Fe(CN)_6^{4-}$	white	2.6
ClO^-	white	2.4
I^-	white	2.8
$[Fe(CN)_5(NO)]^{2-}$	white	28.6
S^{2-}	black	0.4
SO_3^{2-}	white	4.[illegible]
$S_2O_5^{2-}$	white	7.1
SCN^-	white	7.3
$S_2O_3^{2-}$	white	11.1

a— Reduction to silver metal. *b*— Limit of identification in micrograms per 0.04-ml. drop. *c*, *d*, *e*, *f*, and *g* — Values for KOCN, KCN, Na_2S, $Na_2S_2O_6$, and Na_2SO_3 solns. of pH 10.5, 11.5, 15.0, 3.0, and 9.1, respectively. Neutralization affords the same test color, but the sensitivity is reduced.

Preparation of Ag_2CrO_4 Test Paper. Immerse 1-in. wide strips of chromatographic paper (e.g., 3MM Whatman) in 0.01*M* K_2CrO_4 and dry. Next immerse the paper in 0.01*M* $AgNO_3$ and dry. (The deposition of Ag_2CrO_4 on the paper should be as uniform as possible.) Wash the resulting paper with mineral-free water, dry, and cut into 1-in. squares. Since the paper is weakly photosensitive, store it in a tightly closed, amber glass bottle. In all operations with the paper, use tweezers since sufficient salts are present on the fingers to yield a positive reaction.

Test Procedure. With dilute HNO_3 or aq. NH_4 adjust the test soln. approximately to neutrality, using universal pH paper or litmus paper. Spot one drop of the neutral test soln. on a square of the Ag_2CrO_4 paper. The formation of a white, yellow or black spot constitutes a positive test (see Table I for correlation of color with anion).

Discussion & Remarks. Adjustment of the test solution to approximate neutrality is necessary since silver chromate is soluble in acids and aqueous ammonia and is converted to silver oxide by alkali metal hydroxides. Of the metal ions forming insoluble chromates, only mercury and lead interfere. Inorganic anions found not to interfere include arsenate, bromate, carbonate or hydrogen carbonate, chlorate, chlorite, dichromate, fluoride, fluosilicate, hypophosphite, iodate, metavanadate, nitrate, nitrite, orthophosphate, perchlorate, perrhenate, pyrophosphate, selenate, silicate, sulfate, tellurate, tetraborate, and tungstate. No interference was found for acetate, benzoate, citrate, oxalate, salicylate, and tartrate. Hydrogen peroxide gives no color reaction in the test.

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[Rec'd 18June64]

Chemist Analyst
pp. 110-111 ; 1964
54(4)

BIOCHIMICA ET BIOPHYSICA ACTA
126(1):28-36 (1966)

BBA 45375

THE EGRESS OF OXYGEN FROM SHEEP ERYTHROCYTES AFTER MIXING WITH SODIUM DITHIONITE

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(Received February 24th, 1966)

SUMMARY

Spectrophotometric measurements, using the rapid-mixing technique, have been made of the initial rate of egress of oxygen from HbO_2 in sheep erythrocytes after mixing with dithionite. This rate is 18 $sec^{-1} \pm 20\%$ at 25° and the activation energy is 14 kcal $\pm$ 20% for HbB. No variation was found with a change of oxygen concentration from 0.075–0.35 mM at 10°, nor with a change of $Na_2S_2O_4$ concentration from 4–12 g/l. The rate of dissociation was found to be greater, during the later part of the reaction, with the stopped-flow system as compared with the constant-flow technique. An analysis of the results suggests that the cell membrane has negligible resistance to the egress of oxygen and that the initial rate is determined by the chemical reaction of $HbO_2 \rightarrow Hb$. It is also suggested that the internal contents of the erythrocyte may be effectively mixed.

INTRODUCTION

Recent observations by SIRS[1,2] on the rate of egress of oxygen from HbO_2 in erythrocytes, after rapid mixing and 1:20 dilution in an oxygen-free medium, have suggested that, at least during the initial 15% of dissociation, the rate is determined only by the chemical reaction rate. The resistance of the erythrocyte membrane to the passage of O_2 was negligible and the diffusion coefficient for O_2 through the internal haemoglobin was at least an order of magnitude greater than had previously been estimated[3,4]. It was not possible with this technique to assess an upper limit to the diffusion constant and, as has been suggested by PROTHERO AND BURTON[5], it may be that the internal contents of the red blood cell are effectively stirred. The calculations of ROUGHTON[6] however, using the volume of the haemoglobin molecule and the number of molecules present within the volume of the erythrocyte, suggest that they are so closely packed together that rotational but not translational motion is possible. It would appear that mixing oxygenated cells with a reducing agent, which maintained the external oxygen concentration at zero, affords a means of resolving this question. If, as has been indicated, the membrane resistance is negligible and the internal contents are efficiently stirred, when the external oxygen concentration is zero it must be zero throughout the cell interior also. Under these circumstances no

chemical back-reaction of O_2 + Hb can occur and the rate would then be limited only by the chemical rate of dissociation of HbO_2 (ref. 1).

The data on this process so far reported in the literature[6–8], have been obtained using $Na_2S_2O_4$ and broadly indicate that efficient mixing of the internal contents does not occur. In detail, however, not one of these publications agrees with another and in turn they are not consistent with the data obtained by the dilution technique[1]. The differences are most likely to be associated with the use of $Na_2S_2O_4$, which is known to react with haemoglobin in solution[9,10], though with erythrocytes the cell membrane is impermeable to dithionite and prevents this[7,11]. In the present experiments more thorough controls have been used in an attempt to resolve this problem and obtain further information about the degree of internal stirring.

METHODS

The rate of egress has been measured spectrophotometrically, by observing the change of HbO_2 with time, using the rapid-reaction techniques of SIRS AND ROUGHTON[12]. The sheep blood was collected by venepuncture, heparin being used to prevent clotting. The blood was obtained mainly from a cross-bred Suffolk ram with HbB (ref. 13), and a few experiments were made with erythrocytes containing HbA from a Border–Leicester ewe. The whole blood was diluted in a modified form of Ringer–Locke solution in which the bicarbonate concentration was increased to buffer the acidic products of dithionite. The solution with no dithionite consisted of 7.6 g NaCl, 2 g $NaHCO_3$, 0.42 g KCl and 0.24 g $CaCl_2$ per l. A typical solution with dithionite was 5.36 g NaCl, 2 g $NaHCO_3$, 0.42 g KCl, 0.24 g $CaCl_2$ and 4 g $Na_2S_2O_4$ per l. Care must be taken in making up these solutions to avoid precipitation of $CaCO_3$. This was accomplished by equilibrating the solution with 3 cm Hg pCO_2 prior to adding the $CaCl_2$, all other components having previously been dissolved. For the dithionite solution, a stock solution, excluding $Na_2S_2O_4$ and $CaCl_2$, was prepared. A given volume of this was then de-aerated and equilibrated with 3 cm pCO_2 and oxygen-free N_2. Appropriate quantities of $CaCl_2$ and $Na_2S_2O_4$ were weighed and placed into a bottle which was evacuated and to which 3 cm pCO_2 pressure was then applied. The de-aerated stock solution was added, care being taken to exclude contamination with air or oxygen.

The temperature of the reacting mixture was measured to $\pm$ 0.2° by a thermocouple inserted just beyond the observation point. The pH of the solution was checked with a glass electrode and standard pH meter. The oxygen concentrations were determined with the Van Slyke gasometric technique. With the dilute cell suspensions used it was found to be more convenient to centrifuge the red blood cells down and then measure the oxygen concentration of the supernatant. Checks on this method disclosed no significant error. Small samples were taken directly from the cell suspensions and centrifuged to ensure the absence of haemolysis. Microscopic examination was also made in case crenation of cells had occurred.

RESULTS

Though the measurements of the egress of oxygen from haemoglobin in solution are open to criticism, because of the interaction of the products of $Na_2S_2O_4$ and oxygen

with haemoglobin, there was reason to believe that this did not occur with erythrocytes. The initial observations on the egress of oxygen from the erythrocytes after mixing with dithionite were, however, inconsistent, particularly at different wavelengths of light absorption. The decisive experiment which resolved this problem was to mix a 1:100 reduced cell suspension first with de-aerated modified Ringer–Locke solution and then with a similar solution containing dithionite in the constant-flow apparatus. With light filters of 432 mμ and 480 mμ, the dithionite-mixed cells appeared to be more reduced than reduced cells, and with 560 mμ and 542 mμ filters they appeared to be slightly oxygenated. The same difference was obtained at 2 cm and 15 cm from the mixing point, indicating that this was not a chemical effect. There was also no possibility of oxygen being present. The discrepancy would appear to be due to optical inhomogeneities between the dithionite solution and other suspending media. Measurements made with a spectrophotometer indicated that dithionite solutions have a small but definite optical absorption relative to water or Ringer–Locke solution. The addition of oxygen to the solutions increases the dithionite absorption further. This factor on its own, however, is not sufficient to account for all the difference observed and it is probable that a change of refractive index is also involved. Because of this effect, and even with the control precautions given below, the absolute accuracy of the results is of the order ± 15 % and in some cases ± 20 %. The relative error between simultaneous experiments involving a single factor such as oxygen concentration or temperature is ± 10 %. Though this is in some respects unsatisfactory, it may be added that the broad conclusions, based on the dithionite experiments, are in agreement with the dilution technique[1].

Constant-flow measurement

Once the difference of optical homogeneity of the $Na_2S_2O_4$ solutions was appreciated, an investigation was made to find under what conditions the effect would be minimised. No general solution was found that would meet all criticisms. At higher cell concentrations, however, the results proved consistent at wavelengths longer than 480 mμ. A typical experiment is illustrated in Fig. 1. A rather involved procedure was

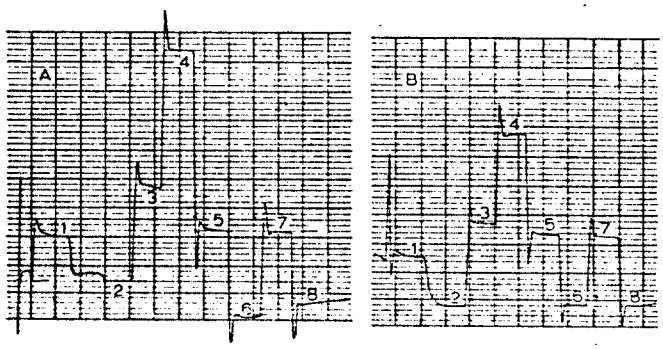


Fig. 1. Direct copy of the records obtained at (A) 432 mμ and 480 mμ and (B) 560 mμ and 480 mμ with the constant-flow system. Temperature 23°; distance from the mixing point 5 cm; flow rate 218 cm·sec^{-1}. Sequence of recording (1) x % HbO_2 cells; (2) reduced Hb cells, no $Na_2S_2O_4$; (3) reacting mixture: reduced cells + HbO_2 cells equilibrated with 4 cm Hg pO_2, no $Na_2S_2O_4$: uptake O_2; (4) HbO_2 cells; (5) HbO_2 cells + reduced cells in a medium containing 4 g/l $Na_2S_2O_4$: egress O_2; (6) reduced cells in a $Na_2S_2O_4$ medium; (7) repeat of (5); (8) outlet flow of (7) suddenly stopped, giving reacted egress point.

used to provide a check on the dithionite difference and enable a calibration of $\%HbO_2$ against deflection to be obtained. All the solutions contained red blood cells equivalent to 1 ml of whole blood in 100 ml of suspension. Four suspensions were used: (a) reduced cells, 0 % HbO_2, (b) cells equilibrated with 4 cm partial pressure air, x % HbO_2, (c) cells equilibrated with 4 cm pO_2, 100 % HbO_2, (d) blood which had been initially reduced and then diluted with an isotonic solution containing 4 g/l $Na_2S_2O_4$. All solutions were equilibrated with 3 cm pCO_2 and maintained at pH 7.4. The exact percentage saturation of the suspension equilibrated with 4 cm air was not determined, as its function was simply to provide a constant reference between 0–100 % HbO_2, in order to check the sensitivity and calibration at different points along the observation tube. The sequence of the experiment is given in the legend to Fig. 1. The mixture of oxygenated cells with reduced cells, containing no $Na_2S_2O_4$, provided a series of points to determine the initial deflection of the unreacted mixture. At the end of each series of measurements the outlet was suddenly closed, producing essentially stopped-flow conditions, so that the reacted base line of reduced cells suspended in a medium containing the reaction products of oxygen and $Na_2S_2O_4$ could be compared with the previous deflections for reduced cells with and without dithionite. It can be seen that with the light filter combination of 560 mμ and 480 mμ these latter points agree. Similar agreement was obtained in this experiment with 560 mμ and 542 mμ filters. However, the third simultaneous experiment at 432 mμ and 480 mμ revealed the discrepancy due to the presence of dithionite. At a lower dilution 1 part of whole blood in 250, a difference between these three reduced suspensions was found at all wavelengths. The reaction curves obtained, with 560 mμ and 480 mμ filters, at a series of points along the observation tube are shown in Fig. 2. Though the

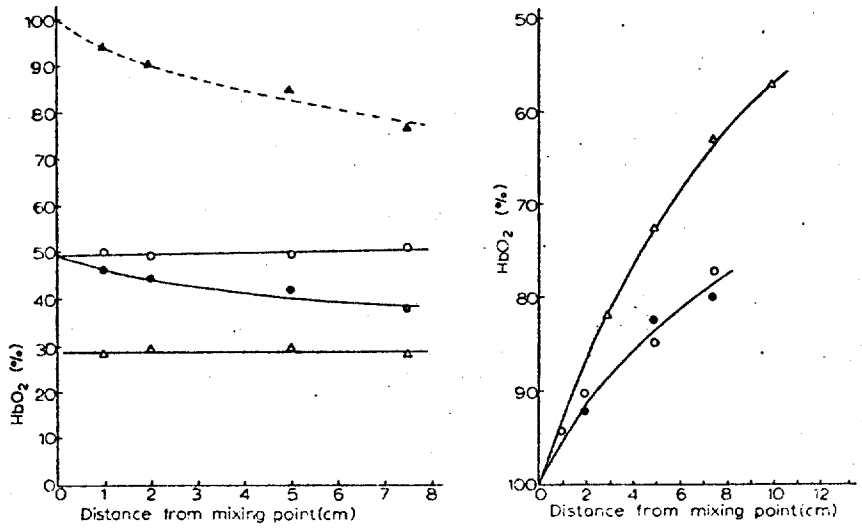


Fig. 2. Complete curve obtained at various points along the observation tube from records Fig. 1B with HbA red blood cells. Initial rate $r = 10.7$ sec^{-1}. △, x% (1); ●, (5) and (7); ○, uptake (3); broken curve, egress points calculated on 100 % scale.

Fig. 3. Comparison of the rate of egress for HbA and HbB in erythrocytes, obtained by the constant-flow, cell-cell procedure at pH 7.4. △, HbB cells, flow rate 232 cm·sec^{-1}, temperature 21.4°, $r = 15$. Circles represent results obtained with HbA cells, flow rate 218 cm·sec^{-1}; temperature 23°, $r = 10.7$. ●, at wavelengths 560 mμ and 542 mμ; ○, at 560 mμ and 480 mμ.

agreement between the deflections of the three reduced suspensions is strong support for the validity of this curve, it still could be that the optical absorption conditions are such that the difference balances out under reduced conditions but not with the oxygenated form. This point, however, is reasonably covered by the agreement between the initial unreacted point as obtained by extrapolation of the uptake curve with no $Na_2S_2O_4$ and the egress values. If allowance is made for the difference at 432 mμ and 480 mμ as being due to a physical shift, a correction can be applied to the 100 % HbO_2 reference points, obtained with a cell suspension without $Na_2S_2O_4$, to calculate the egress points. The initial rate obtained is 10 % higher than at 480 mμ and 560 mμ. Both HbA and HbB cells have been investigated, as is shown in Fig. 3.

Stopped-flow measurement

In principle the difficulty, in the constant-flow experiments, of measuring the % HbO_2 in the cells using reference suspensions of different optical homogeneity, is avoided with the stopped-flow procedure. The curves obtained with this method utilize the same suspension throughout the reaction, and the optical homogeneity of the suspending media is constant. To check this, measurements were made of the rate of uptake of carbon monoxide by reduced haemoglobin in erythrocytes in the presence of $Na_2S_2O_4$. The dithionite was added first to the modified Ringer–Locke solution containing CO and mixed with the reduced cell suspension, and then by suspending the cells in a dithionite medium and using a CO solution without dithionite. In both cases the initial rate agreed with the previous values reported by SIRS[14]. This does not avoid the possibility of O_2 and $Na_2S_2O_4$ products interfering with stopped-flow egress observations, though the constant-flow experiments suggest that this is unlikely. The major problem with the egress of oxygen experiments, however, particularly as satisfactory agreement could not be obtained with different combinations of light filters, was how to obtain a calibration curve of % HbO_2 against deflection. Once $Na_2S_2O_4$ is added to the solution, as in the stopped-flow reaction, no means exists of obtaining various constant % HbO_2 conditions for equivalent calibration. In order to examine this problem, a series of simultaneous measurements were made at different whole blood dilutions and wavelengths of light filters as indicated in Fig. 4. The pattern of this variation does not agree with that obtained in other experiments without $Na_2S_2O_4$; in particular the rates obtained at less than 1:200 dilution are normally in

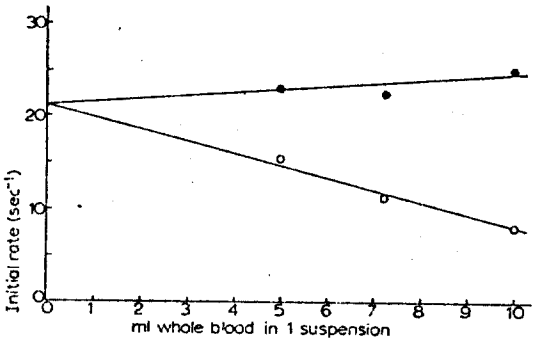


Fig. 4. Stopped-flow observations of the initial rate of egress with variation of cell concentration. Blood taken from the same sample as used in Fig. 2, temperature 26°. ●, 432 mμ and 480 mμ; ○, 560 mμ and 542 mμ.

close agreement. The extrapolated value must therefore be considered to have a wide enough error to include the 1:200 values. A relative comparison, however, at the same cell concentrations and wavelengths, of *e.g.* temperature or oxygen, should not be so affected.

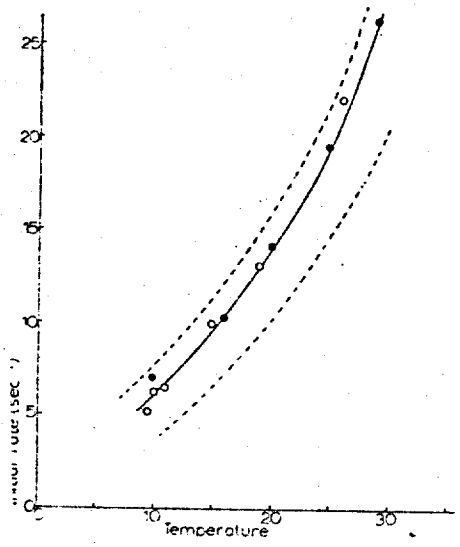
The influence of various factors on the rate of egress

Within the limits of error already discussed, a series of experiments have been performed to investigate the effect of temperature, oxygen concentration, $Na_2S_2O_4$ concentration and time on the initial rate of egress.

The effect of temperature is shown in Fig. 5. The full line represents the mean value as obtained by the extrapolation procedure with the stopped-flow apparatus. The broken curves are the limits of individual values. The higher values were obtained mainly at wavelengths of 432 mμ and 480 mμ and the lower range with 560 mμ and 542 mμ. The results indicated by open circles were obtained on the same cells and under similar conditions, starting at 10° and making measurements at roughly 5° intervals up to 30°, over a period of 3 h.

Experiments to investigate the effect of the initial oxygen concentration within the erythrocyte disclosed no difference. Both at 11° ond 15°, no change of rate was observed for a change of oxygen concentration from 0.075 mM to 0.35 mM. Less systematic experiments at room temperatures (20°–25°) indicate a similar independence. A change of $Na_2S_2O_4$ concentration from 4 g/l to 12 g/l had no significant effect.

The reason why most of the investigations on the effect of oxygen concentration were made at 10°–15° was to minimise any possibility that metabolic activity was involved. Unlike the rate of uptake of oxygen, however, which depends on the time elapsed after withdrawal of the blood sample, no change of the initial rate of egress has been observed over a period of 5 h after collection, between 10° and 25°. Moreover, the rate of egress does not appear to be dramatically reduced or inhibited at 10°.



ig. 5. The variation of the initial rate of egress with temperature. O, measurements made on the me suspension.

Comparison between stopped-flow, constant-flow and dilution techniques

A further improvement in the stopped-flow system was obtained by combining the $Na_2S_2O_4$ method with the dilution technique[1]. Under these circumstances, immediately after rapid mixing, the $Na_2S_2O_4$ concentration is only reduced from 0.4 % to 0.38 %, but the external oxygen concentration is physically diluted 1:20. This means that the initial phase lag of $Na_2S_2O_4$ removing the oxygen is avoided, and the concentration of O_2 to be removed, and in turn the acidic by-products formed, are minimal. A greater measure of agreement was also obtained for light filter combinations of 432 mμ and 480 mμ as compared with 560 mμ and 582 mμ. With this more accurate procedure a comparison was made of the rate of egress using the constant-flow technique, by the dilution method and $Na_2S_2O_4$ calibration method given above, and the stopped-flow $Na_2S_2O_4$/dilution procedure. The results are indicated in Fig. 6. The rate of egress with the stopped-flow technique follows the chemical rate of dissociation over almost the complete range. Variation occurs from sample to sample but in general no fall away is observed before 50 % desaturation is attained and some reach complete reduction even prior to the end-point of the CO + HbO_2 reaction. This latter point is difficult to prove completely as it is very sensitive to the base line position. Attempts to obtain reliable data with the stopped-flow procedure, with only dilution of the HbO_2 cell suspension, were not satisfactory due to the slow attainment of the reaction end-point and the difficulty of ascertaining the % HbO_2 that this represents.

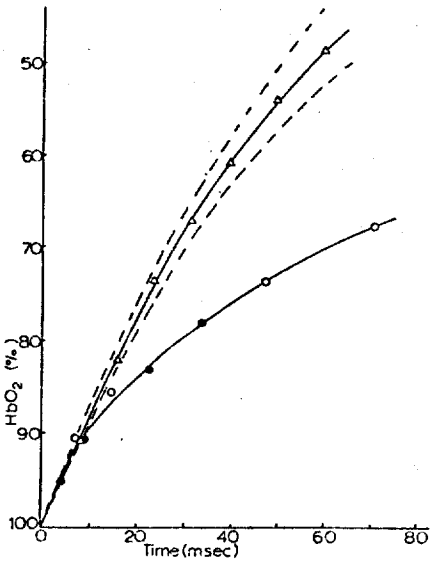


Fig. 6. A comparison of the rate of egress of HbA cells at pH 7.4 and 22.5° ± 0.5° obtained by the constant-flow and stopped-flow methods. △, stopped flow; broken curves indicate limit of error obtained with different light filters and reacted base points. Circles represent constant-flow measurements. ○, dilution only; ●, after mixing with $Na_2S_2O_4$.

DISCUSSION

Consideration of the experimental difficulties of obtaining accurate data when using $Na_2S_2O_4$ is sufficient to account for the variation between the results of previous

workers. The present results, using the constant-flow technique, are similar to those obtained by LEGGE AND ROUGHTON[7]. An analysis of their results by NICOLSON AND ROUGHTON[15] suggested that the red blood cell membrane offered no significant resistance to the passage of O_2. These calculations were made using the half-time for the dissociation process, but because of the difficulties of calibration the initial rate is the more reliable experimental factor. Application of the theory of NICOLSON AND ROUGHTON suggests that the initial rate would vary as the square-root of the oxygen concentration. They emphasise, however, the difficulties of applying the analysis to saturated HbO_2 conditions. The present experimental data indicate that the initial rate is independent of the oxygen concentration, as was found with the dilution technique[1]. The theoretical approach used in this latter case, which implied that the initial rate is determined solely by the chemical dissociation of HbO_2, would therefore appear to be more appropriate. Further confirmation is provided by the variation of this rate with temperature. The activation energy, obtained from the slope of a plot of log rate against the reciprocal of the absolute temperature is 14 kcal $\pm$ 20%, as compared with the value of 16 kcal $\pm$ 10% obtained previously[1].

The slower rate of the constant-flow curves, as compared with the chemical rate of dissociation, at lower % HbO_2 values, would suggest that complete stirring of the internal contents does not occur. The agreement with k_1 over the initial part of the curve can largely be accounted for by the velocity constant k rising as $[HbO_2]$ falls, while as [Hb] increases, k' falls*. The overall effect is that the chemical reaction terms balance each other and stay constant. A theoretical analysis of this situation indicates that the rate of egress would be determined only by the chemical reaction rate constant, though an increase of the diffusion constant is also involved[1]. A comparison of the constant-flow system with the stopped-flow system, however, reveals an interesting discrepancy. While they agree over the initial region of the decay, the stopped-flow curve follows the chemical rate, as determined by k_4, over nearly the whole reaction. The curves were obtained under very similar conditions as regards oxygen concentration, temperature and $Na_2S_2O_4$, so that it is not possible to account for this difference in terms of modification of the chemical factors. It was pointed out by SIRS AND ROUGHTON[12], in their original description of the stopped-flow technique, that the sudden stopping of the solution would be expected to damp down the turbulent eddies within 1 msec if the flow had been only translatory. This would reduce efficient mixing between cells and diffusion in the extracellular fluid would then be a limiting factor. The passive uptake of CO and NO by Hb in erythrocytes agreed according to both methods during the first 50 msec. This was believed to be due to the rotational motion imparted by the tangential mixing jets providing an ancillary source of energy sufficient to maintain a degree of turbulence and mixing over this period. On the basis of measurements of the facilitated uptake of NO (ref. 16) it was proposed that the flexibility of the cell is involved in its respiratory function. More recent experiments, utilizing formaldehyde to make the cells inflexible[17], have confirmed this view. The difference between the egress curves using the constant- and stopped-flow methods could be due to the nature of the flow interacting with a flexible unit. Under turbulent conditions, with micro-eddies smaller than the erythrocyte itself, considerable surface fluctuation could occur but because of the speed of this

* For definitions of these constants, see earlier papers, *e.g.* ref. 2.

process it is unlikely that the cell is completely bent or distorted. *In vivo*, the flow is predominantly laminar and considerable changes of shape and flexing of the red blood cell have been observed. The stopped-flow conditions would more closely resemble this pattern as the more violent eddies rapidly decay. The results are thus compatible with more intracellular mixing occurring in these circumstances than had previously been considered. The flexing of red blood cells has also been shown to induce a degree of turbulence[5] under laminar conditions and this is probably sufficient to prevent diffusion in the extracellular fluid becoming a limiting factor. It is not, however, considered that this implies complete stirring of the internal cell contents in a literal sense. This process could be more efficiently achieved by the movement of water[16] through a relatively immobile suspension of packed haemoglobin molecules. There is an additional implication that if this interpretation is correct, measurements obtained under turbulent conditions with the constant-flow system are likely to underestimate the true rate of transfer. The majority of results previously published are based on measurements of the initial slope where this factor is neither significant, nor thought likely significantly to affect the rates obtained under passive conditions where the cell is considered inflexible. It is interesting to note, however, that the rapid uptakes of NO and O_2 obtained with the constant-flow method show this effect[16]. Even with the stopped-flow system some residual effect of turbulence is probably present. If, as was indicated in INTRODUCTION, efficient internal mixing occurs, the rate would be determined by $k[HbO_2]$ and k rises as the oxyhaemoglobin concentration falls. The dissociation curve would thus be faster during the later part of the decay than that given by the rate constant k_4. This cannot be decisively answered by the present experiments. As has been stated previously, it is likely that the red blood cell functions most efficiently in its natural environment and that the rate of uptake and egress can be adjusted by hydrodynamic fluctuations of the blood flow to suit requirements.

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RAHWAY, N.J., U.S.A.

1968

Wall. Labs. Comm.
23, No. 82 (1960)

The Dithionite—Ascorbate Complex as an Antioxidant for Beer*

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Introduction

Sodium Dithionite** is the salt of one of a group of possibly 12 or more oxy-acids of sulfur. Some of these acids are quite common and others are less widely known because of their instability or rare occurrence. A list of the anions of some of these acids with their formulae is shown in Table I.

TABLE I

LIST OF FORMULAE OF THE ANIONS OF SOME OXY-ACIDS OF SULFUR

Formula	Name
$=SO_2$	Sulphoxylate
$=SO_3$	Sulfite
$=SO_4$	Sulfate
$=S_2O_2$	Thiosulfite*
$=S_2O_3$	Thiosulfate
$=S_2O_4$	Dithionite
$=S_2O_5$	Pyrosulfite
$=S_2O_6$	Dithionate
$=S_2O_7$	Pyrosulfate

*Unstable; known only in form of esters.

From the position of the dithionite anion in this tabulation it is seen that it is closely related to pyrosulfite, the common "metabisulfite" (KMS) which is widely used in the fermentation industries. Pyrosulfite is formed by heating the acid sulfite ($-HSO_3)_2$, to drive off a molecule of water forming ($= S_2O_5$). On solution in water the pyrosulfite anion (S_2O_5), rehydrates and reverts back to sulfite.

While the formulae of dithionite and pyrosulfite indicate a close relationship, the properties of the dithionite anion are strikingly different from either the pyrosulfite or the sulfite anion. The dithionite ion is an unusually strong reducing agent and displays the unique property that when it is dissolved in an aqueous medium it rapidly reacts and combines with molecular oxygen. None of the other stable anions of the sulfur-oxy acids have this property. Neither sulfites nor thiosulfates, commonly thought of as reducing agents, react in this way with oxygen with the order of magnitude shown by dithionite.

As suggested in a recent paper (1), the structure of the dithionite ion can be regarded as a pair of SO_2 units linked by a weak disulfide (S—S) bond as shown in Figure 1. The reducing action of dithionite can be visualized as proceeding by the loss of an electron to any suitable acceptor (oxygen) with the formation of sulfur dioxide.

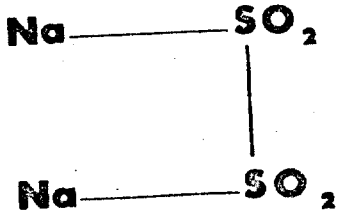

Figure 1
Structural representation of sodium dithionite.

These properties would seem to make the dithionite ion an ideal antioxidant for beer, one that would rapidly react with dissolved oxygen and then disappear and leave only traces of sulfur dioxide in its place. Its value as a beer antioxidant was realized many years ago in a patent granted to Leo Wallerstein (2). Wide use of this invention did not ensue following the issuance of the patent, partly because of the instability of the dithionite ion in dilute solutions, especially at the slightly acid pH of beer.

In dilute solutions the dithionite anion tends to break down with the formation of SO_2 and a precipitate of colloidal sulfur and thereby loses its ability to rapidly combine with molecular oxygen. In the course of our work on antioxidants for beer it was found that the dithionite anion in solution could be stabilized by the presence of ascorbate anions and the loss of its activity and the precipitation be completely prevented. This stabilization is accomplished without any essential decrease in the speed of the oxygen removing capabilities of the dithionite or any effect on the reducing capacity of the ascorbate. The stabilization of the dithionite in solution by ascorbate now made possible the utilization in a thoroughly practical way of the valuable specific antioxidant properties of the dithionite ion. This is the subject matter of a recently issued patent (3).

The effect of the presence of ascorbate ions on the stability of dithionite in solution may be strikingly demonstrated as follows:— Into each of

*Paper presented at the Annual Meeting of the American Society of Brewing Chemists in Minneapolis, and published in the *Proceedings* of the Society, 1960.

**Sodium dithionite is the preferred terminology; one of the names previously used is sodium hydrosulfite.

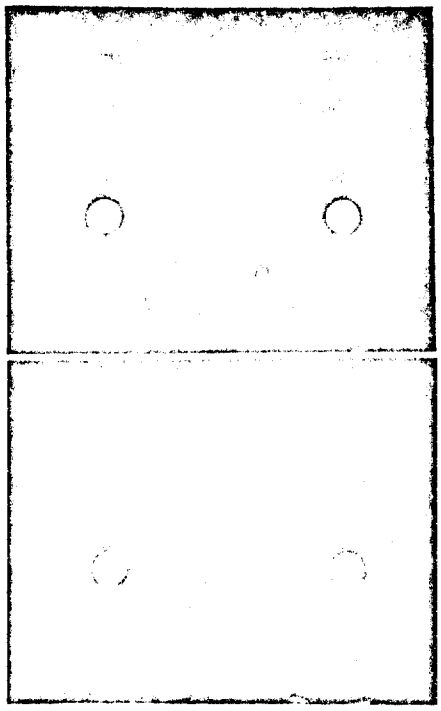

Figure 2

Stability of dithionite solutions. Above, immediately after mixing; below, after 15 min. Left, sodium dithionite; right, sodium dithionite plus isoascorbate.

two dry, stoppered 500-ml Erlenmeyer flasks, add 1.2 grams of sodium dithionite. To one of the flasks add 3.6 grams of sodium ascorbate or sodium isoascorbate. Add 300 ml of water to each flask, swirl to dissolve the salts and then allow to stand. In a few minutes the solution containing only the sodium dithionite will begin to evolve sulfur dioxide and form a white precipitate of sulfur, while the solution containing both the dithionite and ascorbate will remain unchanged. This demonstration is illustrated in Figure 2. Even under air-free conditions, the solution of the sodium dithionite alone will be found to have lost the major portion of its rapid oxygen-combining capacity, while the solution of the combination retains its antioxidant properties essentially unchanged. Evidently there is some interaction of the dithionite and ascorbates in solution. The use of the term "complex" in the title of this paper refers to this combination.

The course of the reaction between solutions of dithionite and molecular oxygen appears to be dependent somewhat on the concentration of dithionite, the quantity of available molecular oxygen, the pH, temperature and other factors. Under the conditions and concentrations existing in the treatment of beer, one mole of dithionite combines with one-half mole of oxygen according to the following reaction:

$$Na_2S_2O_4 + \tfrac{1}{2}\ O_2 + H_2O \longrightarrow 2\ NaHSO_3$$

Oxygen absorptive capacity

In the tests reported in this paper all the results are based on the use of a mixture containing 25% of sodium dithionite and 75% of sodium isoascorbate. Table II shows the total oxygen combining capacity of the mixture as well as that of its individual components at various levels. The dithionite's oxygen combining capacity represents immediate absorptive effect on the oxygen present in the beer system at the time of the treatment and during processing. The absorptive capacity of the isoascorbate, as given in the table, is merely for comparison of available reducing capacities. The ascorbates absorb oxygen only slowly. Their protective effect on beer is due more to their ability to lower the oxidation-reduction potential of the beer system to very low levels. Thus the ascorbate fraction of the mixture would act more as a reservoir of reducing effect to counter the air entering late in processing or existing in the finished package.

TABLE II

OXYGEN ABSORBING CAPACITY
Na DITHIONITE-Na ISOASCORBATE (25-75) MIXTURE

Treatment Level			Dissolved O_2 Equivalents		
			Na Dithionite	Na Isoascorbate	Total
lb/100 bbl	bbl/lb	ppm	ppm	ppm	ppm
1.5	67	60	1.3	3.2	4.5
1.0	100	40	0.9	2.2	3.1
0.8	125	32	0.7	1.7	2.4
0.67	150	27	0.6	1.4	2.0

Under usual brewery conditions when the treatment is applied to prefiltered beer early in processing, the amount of dithionite to be added is in excess of the amount of dissolved oxygen present and hence the dissolved oxygen drops to zero levels. The dissolved oxygen present in the beer may be conveniently determined by the colorimetric procedure developed in our laboratories in 1938 (4). The unreacted dithionite excess does not quickly disappear in the cold storage beer. Any excess is therefore available to react with further introductions of molecular oxygen and this is more fully discussed in a later section of this paper.

In case the dissolved oxygen level is higher than that capable of being handled by the dithionite, then the dithionite will reduce the dissolved oxygen level by stoichiometric reaction, and the effects of any excess oxygen is taken care of by the reservoir of sodium isoascorbate.

Sulfur dioxide levels

In the reaction of dithionite with the dissolved oxygen in beer the dithionite anion is converted into the bisulfite anion. Therefore slight increases

in the trace levels of sulfur dioxide existing in beer can be expected as a result of the treatment. Sulfur dioxide is normally introduced into beer by the fermentation process. Sulfites also have a long tradition of use in the fermentation industries. The increases encountered through the use of dithionite at the recommended treatment levels have not resulted in changes in beer character. Where brewery additions of sulfite are now practiced, then, in most cases, they can be dispensed with or reduced when the dithionite is added.

TABLE III

SULFUR DIOXIDE CONTENT OF BEERS WITH ADDED Na DITHIONITE — Na ISOASCORBATE MIXTURES

Treatment level		"Free" Sulfur Dioxide		"Total" Sulfur Dioxide	
lbs/100 bbl	ppm	Found ppm	Added ppm	Found ppm	Added ppm
none	none	0.3	—	4.8	—
0.4	16	0.6	0.3	7.2	2.4
0.8	32	0.8	0.5	9.1	4.3
1.2	48	1.4	1.1	13.1	8.3
1.5	60	1.8	1.5	14.0	9.3
2.0	80	2.1	1.8	19.4	14.6
Average 1.0	40	—	0.8	—	6.3

Table III shows the results of measurements of the "free" and "total" sulfur dioxide content of pilot brewery beers to which the dithionite-isoascorbate mixture was added. The colorimetric sulfur dioxide method, as published from our laboratories in 1957 (5), was used for these tests. For a full discussion of the significance of "free" and "total" sulfur dioxide in beer, the original paper should be consulted. In this test an average increase of 0.8 ppm of "free" sulfur dioxide and 6.3 ppm of "total" sulfur dioxide resulted from the use of the antioxidant mixture at levels of 40 ppm.* The levels of sulfur dioxide drop off on storage as can be seen from the results in Table IV. The falling-off is slow but definite and seems to be accelerated by the presence of the isoascorbate.

*Equivalent to 1 lb per 100 barrels.

The dithionite anion in beer

If the level of addition of the dithionite-isoascorbate mixture is such that more dithionite is added than is stoichiometrically required to react with the dissolved oxygen at the time of addition, then excess dithionite anions will be temporarily present in the beer. These excess anions will be available for reaction with any further oxygen that may enter the beer during the processing, such as in pumping to the bottle shop and filling into the packages.

The rate of disappearance of the labile dithionite anion in beer has been found to be nicely adjusted to the usual brewery operating temperature conditions. The dithionite is reasonably stable at the cold beer storage temperatures, but quite rapidly changes to sulfite at slightly higher temperatures. Thus there can be no possibility of dithionite residues in the beer following packaging.

The dithionite anion can be specifically determined with high sensitivity by its reaction with certain oxidizing dyestuffs. In a manner similar to its highly characteristic reaction with molecular oxygen, it will also react with these dyes to decolorize them in solution. By careful choice of dyestuff the procedure can be made selective for the dithionite anion. Other beer constituents including isoascorbate and sulfites do not interfere. Indigo disulfonate is a typical dyestuff that answers these specifications.

The actual procedure of the determination is quite simple. The beer is sampled under strictly anaerobic conditions, preferably in a colorless bottle equipped with a special crown so that the dye can be injected without unsealing and exposure to air. A known amount of a dilute solution of the dyestuff is injected and the sample bottle allowed to stand for about an hour at 30°C. At the end of this time, the extent of the decolorization of the added dye is determined, either visually by comparison with standards, or by photometric equipment. By adjusting the concentration of the injected dyestuff, the sensitivity and range of coverage of the technique can be changed as required.

TABLE IV

EFFECT OF STORAGE ON SULFUR DIOXIDE CONTENT OF BEERS

Added Na Dithionite-Na Isoascorbate ppm	Air Content of Bottles ml/12 oz	"Free" Sulfur Dioxide			"Total" Sulfur Dioxide		
		Initial ppm	1 month ppm	2½ months ppm	Initial ppm	1 month ppm	2½ months ppm
None	1	1.3	1.0	0.7	13.4	8.0	7.3
40	1	4.6	3.0	1.6	19.6	14.0	12.1
None	6	1.2	nil	nil	11.5	2.0	2.0
40	6	5.1	1.0	0.2	17.9	8.0	6.1

TABLE V

THE DITHIONITE ANION IN BEER OF DIFFERENT TEMPERATURES

Beer	Added* Sodium Dithionite ppm	Storage Temperature °C	Residual Sodium Dithionite Found: Immediately after Addition ppm	After 1 day ppm	After 2 days ppm	After 3 days ppm	After 1 week ppm
Unpasteurized Prefiltered Beer							
A	29	0	9	8.5	7.5	6.5	4.0
A	29	10	9	5.0	1.5	0.0	0.0
A	29	30	9	0.0	0.0	—	—
B	29	0	11	9.0	8.0	6.5	5.0
B	29	10	11	5.0	1.5	0.0	0.0
B	29	30	11	0.0	0.0	—	—
C	39	0	14	13.0	11.0	10.5	7.5
C	39	10	14	9.0	5.0	0.0	0.0
C	39	30	14	0.0	0.0	—	—
Pasteurized			Immediately after Pasteurization				
A	29		0.0	0.0			
B	29		0.0	0.0			
C	39		0.0	0.0			

*Added as a mixture of Na Dithionite-Na Isoascorbate (Isona) 25 + 75.

The results of tests on the disappearance of the dithionite anion in beer held at different temperatures are contained in Table V. Except at near freezing temperatures, there is a rapid loss of the excess dithionite anions in beer. In fact, in order to have enough dithionite to measure in these experimental beers, we had to add much more than would normally be used.

The data of Table V have been used to construct the curves of Figure 3. It appears that the unreacted dithionite in beer has a half-life of about a week at cold storage temperature (0°C). At 10°C the half-life is about 1 day, while at room temperature it is only a matter of hours, since no dithionite was found after 1 day at 30°C, the shortest period employed in these tests. The temperature of pasteurization rapidly converted the dithionite to sulfite since no dithionite was found in any beer immediately after pasteurization.

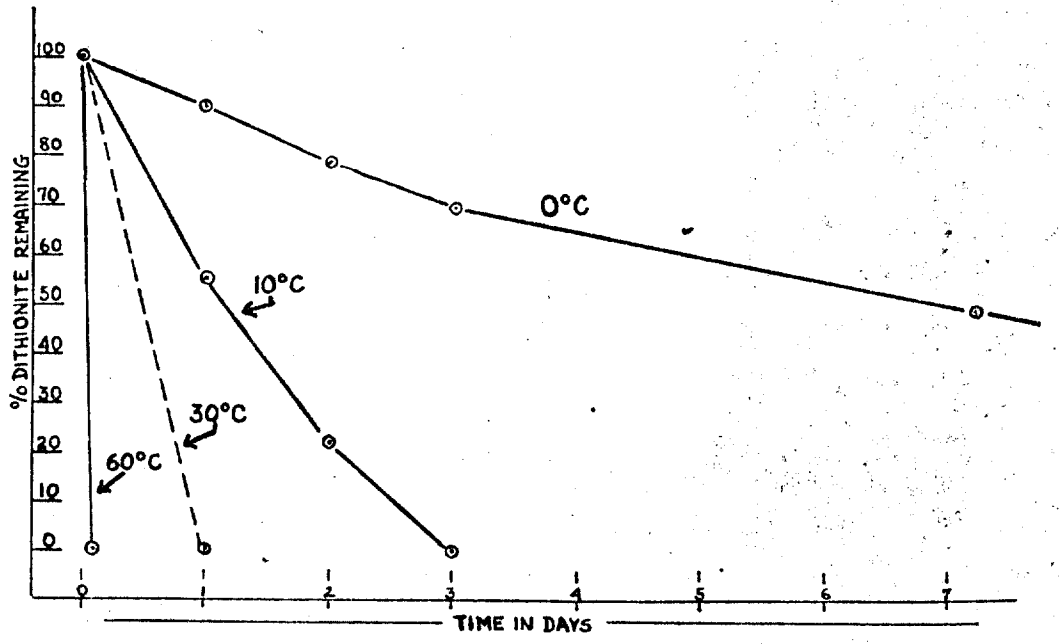

Figure 3
Average time of disappearance of dithionite anions from beer.

Effect on beer color

The dithionite ion has a decolorizing action on the coloring matter in beer, being much more effective than other common reducing agents. As a laboratory demonstration of this effect, the dithionite-isoascorbate mixture was added to beers at several levels and the resulting beer color was determined. These results are contained in Table VI.

TABLE VI

EFFECT OF Na DITHIONITE-Na ISOASCORBATE (25-75) MIXTURE ON BEER COLOR

	Beer Color			
	Treatment Level			
Beer	None	80 ppm	40 ppm	27 ppm
A	4.1	3.4	3.7	3.7
B	3.6	2.9	3.1	3.2
C	3.1	2.6	2.8	2.9
D	2.7	2.2	2.3	2.4

Additional results illustrating the drop in color values of beers treated under pilot brewery conditions and in large scale breweries will be found in Tables IX and X.

Effect on diacetyl

The occurrence of diacetyl and its effect on beer was the subject of a paper presented before this Society in 1952 by West, Lautenbach & Becker (6), who reviewed the literature and noted the close oxidation-reduction relationship between the relatively inodorous acetyl methyl carbinol and the stronger butter-flavored diacetyl. These workers also indicated that the taste threshold for diacetyl in beer was about 0.3 ppm. In a second paper, Burger, Glenister and Becker (7) found that exposure to air during processing tends to predispose the beer to development of a diacetyl flavor. They also showed that treatment of the beer with active yeast removed the off-taste, presumably by chemical reduction of the diacetyl. The reducing agents potassium metabisulfite and ascorbates, it is stated, were only partly effective in removing diacetyl. Packaged beers with low ITT values can lose a fraction of their diacetyl during storage.

Since it appeared that a strong reducing enzyme system lowers the diacetyl content, we tried the dithionite-ascorbate mixture on beers containing added diacetyl. These tests were conducted in the laboratory on 12-ounce bottles of beer and the chemical determination of the diacetyl content before and after treatment. Our initial results were rather negative at low treatment levels until we went to levels of about 40 ppm and over, where we found nearly complete elimination of the diacetyl. This we attributed to the experimental conditions inherent in working with small quantities of beer; with the lower levels of treatment all of the dithionite was used up reacting with the dissolved oxygen and no free dithionite ions were available for reaction with diacetyl. In large-scale brewery treatments there should be a corresponding economy of the dithionite ions making for greater efficiency of diacetyl reduction at lower treatment levels. When precautions were taken to avoid contact with air, the laboratory test results shown in Table VII were obtained. The diacetyl was determined by the procedure of reference 6.

TABLE VII

EFFECT OF Na DITHIONITE-Na ISOASCORBATE ON DIACETYL IN BEER IN LABORATORY BOTTLE TESTS

	Diacetyl — ppm		
Time After Treatment of Sample	Control	NaD-NaIsoAA 40 ppm	NaD-NaIsoAA 27 ppm
24 hours	1.15	0.29	0.51
6 days	1.25	0.26	0.45
2 months	1.20	0.02	0.45

TABLE VIII

EFFECT OF Na DITHIONITE-Na ISOASCORBATE ON DIACETYL IN BEER IN THE PILOT BREWERY

	Diacetyl — ppm			
		NaD-NaIsoAA		NaIsoAA
Sample	Control	65 ppm	32 ppm	80 ppm
Immediately after addition	1.00	0.25	0.60	1.00
After filtration, before carbonation	0.90	0.25	1.00	1.15
After carbonation	1.05	0.29	0.80	1.35
Bottled				
Pasteurized	1.20	0.28	0.80	0.80
Stored 2 weeks	1.10	0.25	0.90	1.30
Stored 4 weeks	1.20	0.30	0.75	0.85

The results of a typical pilot brewery run, in which high dissolved oxygen levels were encountered, are given in Table VIII. Taste tests were also conducted. The samples treated with the dithionite-isoascorbate mixture at 65 ppm were free of the diacetyl off-flavor while the other samples had a strong diacetyl flavor.

Pilot brewery tests

After the initial laboratory development, tests on the efficacy of the dithionite-isoascorbate mixture were conducted in our pilot brewery preliminary to large-scale tests in breweries. Our general procedure was to take regular brewery beer, just after fermentation (received in half barrels), transfer it to a tank and determine the dissolved oxygen content. If too low for the purposes of the tests, the dissolved oxygen was adjusted to the desired level by controlled bubbling of air through the beer. This beer was then transferred to the 3-gallon storage tanks and the various treatments were applied. The dissolved oxy-

TABLE IX

RESULTS OF PILOT BREWERY EXPERIMENTS

Treatment	ppm	Dissolved Oxygen Content					Examination of Finished Beer		
		Before Treatment ppm	After Treatment: After 1 Hour ppm	After 18 Hours ppm	After Filtration ppm	After Carbonation ppm	Sulfur Dioxide ppm	Color	
Test No. 30									
Control I	—	0.6	0.6	0.7	1.4	0.7	8.0	3.0	
Control II	—	0.7	0.7	0.7	1.0	0.6	8.2	3.1	
KMS (7 ppm SO_2)	—	0.6	0.6	0.7	1.3	0.7	14.8	3.0	
NaD-NaIsoAA	80	0.6	0.0	0.0	0.0	0.0	17.7	2.5	
NaD-NaIsoAA	40	0.6	0.0	0.0	0.2	0.1	12.0	2.8	
NaD-NaIsoAA	27	0.7	0.1	0.2	0.5	0.1	11.3	2.9	
NaIsoAA	80	0.6	0.6	0.7	1.0	0.4	8.0	2.8	
NaIsoAA	40	0.6	0.7	0.7	1.1	0.5	7.3	2.9	
NaIsoAA	27	0.7	0.7	0.7	1.0	0.4	8.2	2.9	
Test No. 37									
Control I	—	0.35	0.30	0.30	0.50	0.30	2.5	3.1	
Control II	—	0.35	0.30	0.30	0.60	0.40	2.9	3.1	
NaD-NaIsoAA	32	0.35	0.0	0.0	0.20	0.10	7.0	2.8	
NaD-NaIsoAA	27	0.35	0.0	0.0	0.30	0.15	6.3	2.9	
NaIsoAA	40	0.35	0.30	0.30	0.80	0.40	3.2	3.0	
NaIsoAA	32	0.35	0.25	0.25	0.60	0.30	2.4	2.9	

gen levels were determined before and after treatment and at intervals during storage. The beers were then processed by our usual pilot brewery procedure. The finished beer was bottled, the air contents of the bottles adjusted and after capping, the bottles were pasteurized. The sample bottles were then used for various tests.

Table IX contains results of the dissolved oxygen tests typical of the many runs conducted in the pilot brewery. The addition of the dithionite-isoascorbate mixture to the beer causes an immediate lowering of the dissolved oxygen content. Zero levels of dissolved oxygen are easily attainable in the treated beer, depending upon the initial dissolved oxygen levels and extent of the treatment. The dissolved oxygen levels of the beers treated with the dithionite-isoascorbate mixture remain at lower levels than the corresponding controls throughout the entire cellaring and processing operations. The pilot filtration procedure permits some pickup of air, as can be seen by the increases noted on the "after filtration" samples.

A substantial drop in the dissolved oxygen content is noted in all the samples after carbonation. This is due to the particular conditions operating in our pilot carbonating equipment, where a rather prolonged carbonation and blow-off tends to sweep out the dissolved air. The beers treated with the isoascorbate alone show dissolved oxygen contents similar to the controls because the ascorbates absorb oxygen only slowly. The isoascorbate, of course, has retained its reducing activity when admixed with the dithionite as is evidenced by the low ITT values of the treated beer.

Included also in the tabulation are the results of the examination of the beers for "total" sulfur dioxide, ITT and color. The "total" sulfur dioxide content of the beer increases in proportion to the amount of dithionite added. 40 ppm of 25-75 sodium dithionite-sodium isoascorbate mixture will theoretically raise the "total" sulfur dioxide content by 7 ppm. This is a maximum level as the amount found by analysis of the beers is generally slightly less than this.

The storage stability of the enzymatically chill-proofed pilot beers as measured by the rate of development of chill haze is dependent on the exposure to oxygen during processing, the air content of the finished package and the level of treatment. When the exposure to dissolved oxygen is kept at a minimum and the "packaging" air is kept very low, there is some improvement in storage stability between the treated and the untreated controls. As the exposure to oxidative conditions in processing increases or the air level in the package goes up, there is correspondingly greater improvement in the physical stability of the treated bottles as compared with the controls. Figure 4 shows the typical curves of the chill haze development in beers having about 0.4 ppm of dissolved oxygen in storage and in one case about 0.5 ml, in the other 3 to 4 ml of "packaging" air per bottle. In these pilot experiments the chill-proofing treatment was used at marginal levels to give a rather poor storage stability in the presence of air so that results will be available without waiting for unduly long periods of time. The positions of the various curves are therefore only relative, thus the absolute values per unit time interval should be disregarded as this will still depending upon innate chillproofness of the particular samples. In the graph, a control untreated sample is compared with a dithionite-isoascorbate treatment at 32 ppm and an isoascorbate treat-

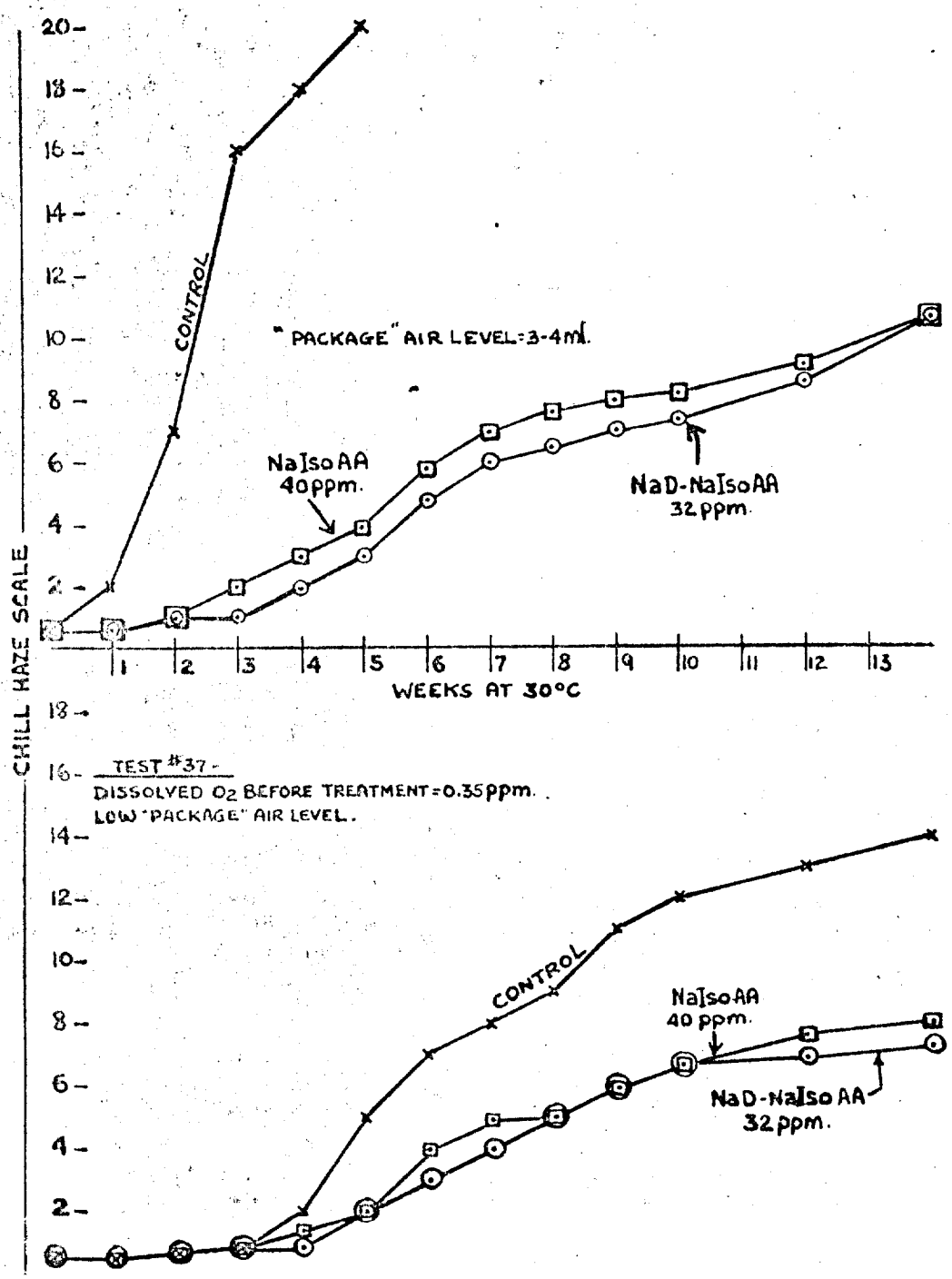

Figure 4

Pilot brewery tests.

ment at 40 ppm. It is seen that the overall effect of the two levels is about the same, indicating a greater efficiency for the combination treatment. Increasing the levels of the treatments would, of course, result in flatter curves and produce more stable beers.

TABLE X

RESULTS OF BREWERY EXPERIMENTS

Brewery	Treatment Added ppm	Dissolved Oxygen Content: Before Treatment, Unfiltered ppm	Before Treatment, Prefiltered ppm	Storage Tank, Initial ppm	Storage Tank, 7 Days ppm	After Final Filtration ppm	Bottling Tank ppm	Examination of Finished Beer: Sulfur Dioxide ppm	ITT	
A	None	0.40	0.70	0.80	0.85	—	1.00	20*	390	
A	40	0.50	0.65	0.00	0.00	—	0.20	12	0	
B	None	0.15	0.50	0.90	1.20	—	1.70	1	500	
B	40	0.15	0.50	0.00	0.10	—	0.20	6	0	
C	None	0.30	—	0.45	0.55	—	0.70	15	800	
C	27	0.30	—	0.00	0.00	—	0.10	19	0	
D	None	0.20	0.20	0.30	0.40	0.5	0.60	25*	200	
D	27	0.20	0.20	0.00	0.10	0.1	0.10	18	0	

*KMS added to control beer; omitted in treated beer.

Large-scale brewery tests

A number of large-scale brewery tests of the sodium dithionite-sodium isoascorbate treatment have been made to obtain confirmatory data under practical operating conditions. The treatment was added by preparing a concentrated solution in a mixture of cold water and beer or cold beer at about one and one-half gallons per pound of mixture. We prefer to have beer present in this concentrated solution because of the surface cover of beer foam which forms and protects the solution from contact with air. The treatment was applied to the prefiltered fermenter beer on its way into storage. The concentrated solution was either proportioned into the beer lines as the storage tanks were being filled (the preferred method) or the concentrated solution was added through the manhole of the tank into a deep layer of beer in the tank and the manhole closed and the beer immediately started running into the tank. The concentrated solutions were prepared immediately before use* to avoid loss in activity due to contact with air while awaiting addition to the beer. When a proportioning device was used, its setting was adjusted so that the full charge of the concentrated solution was completely added to the tank in the first 15 to 30 minutes of flow. The currents caused by the beer entering the tank later were depended upon to form a homogeneous solution. Samples for dissolved oxygen determinations were taken at various points in the processing sequence. Typical results of these tests are shown in Table X.

Inspection of the dissolved oxygen test results shows that rapid and efficient removal of the dissolved oxygen is obtained on the storage beer as a result of the treatment. The treated beers will also enter the packaging lines with substantially lower dissolved oxygen than the corresponding controls.

The analysis of the fininshed beers shows the slight increases in sulfur dioxide levels as a result of the treatment, except in the beers from breweries A and D where KMS was used in the controls and omitted in the treated runs. The level of KMS addition was that normally used and resulted in higher levels of SO_2 than that obtained with the dithionite-isoascorbate treatment. Significant differences were also obtained in the I.T.T. and the beer color; lightening of the beer color results from application of the dithionite-isoascorbate treatment.

Stability of the beer as regards chill haze development and desirable flavor retention is dependent on many factors. These are sometimes difficult to control and replicate under brewery operating conditions. In general the storage stability results on chill haze development followed the same pattern shown for the pilot brewery beers with the exception that the untreated controls were more chillproof than those shown in Figure 4. As the air levels in the package increased, the treatment shows up more effectively. While most breweries try to hold their package air levels at low values, a certain percentage of bottles or cans slip by with higher than desired air contents. It is for this group of packaged beer that the treatment is especially valuable.

Taste tests conducted on the samples from these practical brewery tests by brewery personnel, "expert" panels at our laboratory and a "consumer" type panel composed of our office and laboratory personnel satisfied us that the treatment produced no immediate off characteristics in the beer flavor. Further tests on the beers after storage of the samples indicated less rapid taste changes in the treated samples with a preference for the treated samples by the "consumer" type panels. The evidence of the taste tests from our "expert" taste panel consistently showed the treated samples to be less "harsh," being variously described as "softer, smoother and well rounded." Especially because of the limitation in panel size, there could be no justification for statistical evaluation here of such indications.

*A covered stainless steel mixing tank was used.

Summary

Sodium dithionite is the salt of one of the group of oxy-acids of sulfur that includes sulfite, sulfate, thiosulfate and pyrosulfite (KMS). It has the unique property in solution of rapidly reacting with dissolved oxygen. Its aqueous solutions require stabilization to prevent loss of activity and precipitation of colloidal sulfur. This can be accomplished by the presence of ascorbates. The mixture of sodium dithionite and sodium isbascorbate is used as an improved antioxidant for beer.

When sodium dithionite-sodium isoascorbate is added in the brewery during processing, complete removal of the dissolved oxygen results. The dithionite is converted to sulfite in reacting with the molecular oxygen. When the mixture contains 25% of sodium dithionite and is used at a level of 40 ppm, the increase in "free" sulfur dioxide is small and variable, depending on the complexing power of the beer (usually less than 2-3 ppm); the "total" sulfur dioxide is increased by about 6 or 7 ppm.

Because of the instability of the dithionite ion in beer at above freezing temperatures, no dithionite is found in the finished packaged beverage. When the dithionite ion has disappeared, there is still a reservoir of isoascorbate available for further antioxidant effect in the beer.

Results of pilot brewery and large-scale brewery tests are given and discussed. The dithionite ion has a strong effect in lightening the color of the beer. It also reacts with diacetyl to give reaction products free of odor and any perceptible taste.

ACKNOWLEDGMENTS

The author wishes to acknowledge gratefully the cooperation and help of Mr. S. Scaturo in conducting the pilot brewery and large-scale brewery tests; of Mr. C. Laschiver, Mr. W. Morris and Mr. J. Donnelly in the various phases of laboratory testing; of Dr. S. R. Green for his suggestions and help in the work relating to diacetyl; and of Mr. Philip P. Gray for valuable suggestions relating to the manuscript.

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[Résumé]

Le Dithionite-Ascorbate Complex comme Antioxydant pour la Bière.

Le dithionite de soude (hydrosulfite de soude) est le sel d'un des groupes d'oxy-acides du soufre qui comprend le sulfite, sulfate, thiosulfate et pyrosulfite (KMS). Il a la propriété unique en solution de réagir rapidement avec l'oxygène dissous. Les solutions aqueuses nécessitent la stabilisation pour prévenir la perte d'activité et la précipitation de soufre colloïdal. Ceci peut être réalisé par la présence d'ascorbates. Le mélange de dithionite de soude et de isoascorbate de soude est utilisé comme un antioxydant amélioré de la bière.

Quand on ajoute du dithionite de soude-isoascorbate de soude en brasserie pendant la fabrication, on obtient une totale disparition de l'oxygène dissous. Le dithionite est converti en sulfite par la reaction avec l'oxygène moléculaire. Lorsque le mélange contient 25% de dithionite de soude et est utilisé au taux de 40 ppm, l'augmentation en bioxyde de soufre "libre" est petite et variable, selon le pouvoir complexant de la bière (généralement moins de 2-3 ppm); le bioxyde de soufre "total" est augmenté de 6 à 7 ppm.

A cause de l'instabilité de l'ion dithionite dans la bière à des températures plus élevé que le point de congélation, on ne trouve pas de dithionite dans la boisson finie et soutirée. Lorsque l'ion dithionite a disparu, il y a toujours une réserve disponible d'isoascorbate pour assurer dans la bière l'action antioxydante ultérieure.

Les résultats d'essais en brasserie-pilote et en brasserie industrielle sont donnés et discutés. L'ion dithionite a un effet marqué dans la décoloration de la bière. Il réagit également avec le diacetyl pour donner des produits exempts d'odeur ou de quelque goût perceptible.

[Resumen]

El complejo ditionito-ascorbato como un antioxidante para la cerveza.

El ditionito de sodio (hidrosulfito de sodio) es la sal de un miembro del grupo de los oxiácidos del azufre que incluye a los sulfitos, sulfatos, tiosulfatos y pirosulfitos (KMS). Tiene la singular propiedad de reaccionar rápidamente en solución con el oxígeno disuelto. La solución acuosa necesita estabilizarse para evitar una pérdida de actividad y precipitación de azufre coloidal. Esto se puede realizar en presencia de ascorbatos. La mezcla del ditionito de sodio con ascorbato de sodio se emplea como un antioxidante mejorado para la cerveza.

Cuando se añade ditionito de sodio-isoascorbato de sodio en la cerveza durante el proceso, se obtiene una remoción completa del oxígeno di-

suelto. El ditionito se convierte en sulfito al reaccionar con el oxígeno molecular. Cuando la mezcla contiene 25% de ditionito de sodio y se emplea en la proporción de 40 ppm., el aumento de bióxido de azufre "libre" es pequeño y variable, dependiendo del poder de la cerveza para formar complejos (generalmente es menor de 2-3 ppm); el bióxido de azufre "total" se aumenta de 6 ó 7 ppm.

A causa de la inestabilidad del ión ditionito en la cerveza a temperaturas sobre el punto de congelación, no se encuentra ditionito en la bebida terminada y envasada. Cuando ha desaparecido el ión ditionito, todavía queda una reserva de [illegible] ascorbato disponible para un efecto antioxi[illegible] posterior en la misma cerveza.

Se proporcionan los resultados de las pr[illegible] hechas en planta y en grande escala en cerv[illegible] El ión ditionito tiene un fuerte efecto en d[illegible] nuir el color de la cerveza. También reaccion[illegible] el diacetilo para dar sustancias sin olor y n[illegible] ceptibles al sabor.

[Zusammenfassung]

Der Dithionit-Ascorbat-Komplex als Antioxydans für Bier.

Natriumdithionit (Natriumhydrosulfit) ist das Salz einer der Gruppen der Oxysäuren des Schwefels, zu der Sulfit, Sulfat, Thiosulfat und Pyrosulfit gehören. In Lösung verfügt es über die einzigartige Eigenschaft, mit gelöstem Sauerstoff sehr schnell zu reagieren. Seine wässerigen Lösungen müssen stabilisiert werden, damit ein Verlust an Aktivität und ein Ausfällen von kolloidalem Schwefel verhindert wird. Dies lässt sich mit Hilfe von Ascorbaten erreichen. Die Mischung von Natriumdithionit und Natriumisoascorbat wird als verbessertes Antioxydans für Bier verwendet.

Wird Natriumdithionit-Natriumisoascorbat in der Brauerei während der Bierbereitung zugefügt, so wird der gelöste Sauerstoff vollständig entfernt. Durch die Reaktion mit molekularem Sauerstoff wird das Dithionit in Sulfit umgewandelt. Wenn das Gemisch 25% Natriumdithionit enthält und in einer Menge von 40 mg/l angewendet wird, ist die Zunahme an "freiem" Schwefeldioxyd klein und veränderlich, und zwar in Abhängigkeit vom Komplexbildungsvermögen des Bieres (in der Regel weniger als 2 bis 3 mg/l; der "Gesamt"-Schwefeldioxydgehalt wird um etwa 6 oder 7 mg/l erhöht.

Auf Grund der Instabilität des Dithionit-Ions im Bier bei höheren als Gefriertemperaturen wird im abgefüllten Getränk kein Dithionit mehr gefunden. Wenn das Dithionit-Ion verschwunden ist, dann steht immer noch ein Reservoir an Isoascorbat zu weiterer antioxydativer Wirkung im Bier zur Verfügung.

Es werden die Ergebnisse von Versuchen in der Versuchsbrauerei und im Grossbetrieb wiedergegeben und besprochen. Das Dithionit-Ion hat einen starken Einfluss auf die Aufhellung der Bierfarbe. Es reagiert mit Diacetyl unter Bildung von Verbindungen, die ohne Geruch und frei von irgendeinem wahrnehmbaren Geschmack sind.